43569

National Library of Canada | Bibliothèque nationale du Canada

Canadian Theses Division | Division des thèses canadiennes

Ottawa, Canada
K1A 0N4

PERMISSION TO MICROFILM — AUTORISATION DE MICROFILMER

- Please print or type — Écrire en lettres moulées ou dactylographier

Full Name of Author — Nom complet de l'auteur: LAWRENCE WAYNE THOMSON

Date of Birth — Date de naissance:

Country of Birth — Lieu de naissance: Canada

Permanent Address — Résidence fixe: R. R. 5, Edmonton Alberta

Title of Thesis — Titre de la thèse: Lipid Composition and Acetyl CoA Carboxylase Activity in Developing Leaves and Chloroplasts of Gateway Barley and its Virescens Mutant

University — Université: Alberta

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée: PhD Plant Biochemistry

Year this degree conferred — Année d'obtention de ce grade: FALL 1979

Name of Supervisor — Nom du directeur de thèse: Professor Saul Zalik

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

Date: Oct 18, 1979

Signature: Lawrence Thomson

NL-91 (4/77)

National Library of Canada

Cataloguing Branch
Canadian Theses Division

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

Bibliothèque nationale du Canada

Direction du catalogage
Division des thèses canadiennes

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

NL-339 (3/77)

THE UNIVERSITY OF ALBERTA

LIPID COMPOSITION AND ACETYL COA CARBOXYLASE ACTIVITY IN DEVELOPING LEAVES AND CHLOROPLASTS OF GATEWAY BARLEY AND ITS VIRESCENS MUTANT.

by

Ⓒ LAWRENCE W. THOMSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY
IN
PLANT BIOCHEMISTRY and PHYSIOLOGY

PLANT SCIENCE

EDMONTON, ALBERTA
FALL, 1979

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled LIPID COMPOSITION AND ACETYl COA CARBOXYLASE ACTIVITY IN DEVELOPING LEAVES AND CHLOROPLASTS OF GATEWAY BARLEY AND ITS VIRESCENS MUTANT. submitted by LAWRENCE W. THOMSON in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in PLANT BIOCHEMISTRY and PHYSIOLOGY.

Saul Zalik
Supervisor

Paul R. Gorham

Edwin A. Cossins

J. Brian Mudd
External Examiner

Date Oct. 12, 1979

ABSTRACT

To further explore chloroplast development and to extend the discription of the virescens mutant of Gateway barley the changes in acyl lipids, pigments and acetyl CoA carboxylase activity in developing seedlings and chloroplasts of the mutant (M) and Gateway barley (N) were studied. Apical 3cm leaf segments were extracted with chloroform:methanol, the extracts were purified on Sephadex G-25 columns and the polar lipids were separated on 2D TLC silica gel plates. The pigment remaining on the Sephadex column was identified as flavonoids and a zone on the TLC plates which did not correspond to the usual standards was identified as gramine. Quantification of acyl lipids by either polar head group anelysis or fatty acid analysis using a C:17 internal standard gave similar results. The percent of the total lipid extract that was quantified for the M at 4, 6 and 8 days was 46, 53 and 65% respectively and for the N the values were 60, 61 and 68%. Of these, acyl lipids represented 37, 41 and 48% in the M and 50, 43 and 47% in the N. By 8 days mono- and di-galactosyldiglyceride (MG,DG) accounted for 45 and 25% of the total acyl lipid of both the M and N. For the period of study this represented a 4-fold increase in MG and a 2.5-fold increase in DG in the M but only a 1.8-fold increase for MG and DG in the N. These increases were closely correlated with the increases in chlorophyll. Chlorophyll increased sharply between 4 and 6 days for the N, while in the M it rose from 7% to 50%

relative to the normal by 8 days. The proportions of the various fatty acids were unique for the lipid classes. The only major quantitative change for a fatty acid was for hexadecanoate in phosphatidyl glycerol which increased from 5% at 4 days to 25 to 30% by 8 days. Relative to the N the carotenoid content of the M increased from 14 to 50% between 4 and 8 days. In both the M and N the increases in β-carotene and chlorophyll were closely correlated.

Acetyl CoA carboxylase activity was determined in tissue homogenates and chloroplast preparations. Its activity was analyzed as the acetyl CoA dependent incorporation of ^{14}C-bicarbonate into an acid stable product. The absolute requirement for ATP and $MgCl_2$, the complete inhibition with avidin and end product analyses were consistent with the presence of acetyl CoA carboxylase activity. Little difference in activity was found between M and N tissue homogenates from the 1 to 3 day stages during which period both showed a 3-fold increase. However, by 4 days the activity of the M exceeded that of N. Fractionation studies showed that the enzyme was a soluble protein present in the stromal fraction of chloroplasts. Likewise biotin content was also highest in the stroma, although it was found in the lamellar fraction as well. For both M and N the highest acetyl CoA carboxylase activities were obtained in stromal preparations from 4 day seedlings. These were 54 nmol/mg protein/ min for the M and 31 nmol/mg protein/ min for the N and they represent the first data showing appreciable acetyl CoA carboxylase activity in any chloroplast preparation.

ACKNOWLEDGEMENTS

The encouragement and guidance of Professor Saul Zalik throughout the course of my research program and during the preparation of this manuscript is gratefully acknowledged.

I wish to thank Dr. Jill Williams for kindly monitoring the plastid isolates for bacterial contamination, and Professor Fred Cook for the generous supply of *E. coli* cells.

A special thank you is also extended to Mr. Barry Zytaruk for the calculation of the fatty acid peak areas and for preparing the figures and to Mr. R.N. Swindlehurst for the IR scans. Also I wish to thank Mrs. Lynne Robertson for kindly typing much of the manuscript.

Financial assistance from the National Research Council of Canada and the University of Alberta is acknowledged with thanks.

Table of Contents

Chapter Page

I. INTRODUCTION..1

II. LITERATURE REVIEW...5

A. Overall Structure, Activity and Composition of the Chloroplast.....................................5

Internal Membrane Network..........................5

Plastoglobules...6

Photochemical Activity...............................6

Chemical Composition..................................7

B. Chloroplast Membrane Studies.........................9

Separation of Photosystems.........................9

Lipid Composition of Stroma and Grana Membrane Fragments..............................10

Subfractionation of the Photosystem Complexes..12

C. Chloroplast Membrane Model.........................15

Freeze Fracture Pattern and Chlorophyll Protein Complexes..............................15

Acyl Lipids as Matrix and Boundary Lipids...20

D. Chloroplast Development............................24

Plastid Types..24

Membrane Differentiation...........................26

E. Biosynthesis of Chloroplast Constituents.......33

Plastid Genome..33

Protein Synthesis..36

Chlorophyll Biosynthesis............................38

Carotenoid Biosynthesis.............................41

Acyl Lipid Biosynthesis..........................44

F. Acetyl Coenzyme A Carboxylase..................52

Multienzyme Complexes from Animal and Yeast Tissue......................................52

Escherichia coli Enzyme Complex.............52

Wheat and Barley Embryo Enzyme Complexes....53

Chloroplast Enzyme Complex..................54

Other Chloroplast Enzyme Complexes..........58

III. LIPID CHANGES DURING GREENING OF BARLEY SEEDLINGS.60

MATERIALS AND METHODS.......................60

A. Plant Material.................................60

B. Solvents.......................................61

C. Lipid Extraction...............................61

D. Separation of Polar Lipids.....................63

E. Identification of Phospholipids, Glycolipids and Gramine......................................64

F. Lipid Quantification...........................64

Phosphorus Analysis.........................64

Galactose Analysis..........................65

Internal Fatty Acid Standard................66

G. Chlorophyll Determination......................68

H. Nitrogen Analysis..............................68

I. Gramine Analysis...............................69

J. Analysis of Pigment Remaining on the Sephadex Column.......................................72

K. Carotenoid Estimation..........................73

Extraction..................................73

Thin Layer Chromatography...................74

IV. RESULTS...77

A. Lipid Extractions...............................77

B. Flavonoids......................................78

C. Gramine...79

D. Lipid Identification............................80

E. Lipid Quantification............................87

F. Changes in Lipid Constituents...................87

G. Variations in Fatty Acid Composition of Acyl Lipids...90

H. Carotenoids.....................................97

V. DISCUSSION.......................................104

VI. ACETYL CoA CARBOXYLASE ACTIVITY IN BARLEY SEEDLINGS..115

MATERIALS AND METHODS.........................115

A. Chemicals......................................115

B. Buffers and Reagents...........................115

C. Plant Material.................................116

D. Whole Tissue Analysis..........................117

E. Plastid Isolation..............................117

F. Plastid Counts.................................118

G. Bacterial Counts...............................118

H. Chloroplast Fractionation......................119

I. Partial Purification of Acetyl CoA Carboxylase Subunits from _Escherichia coli_...............119

J. Assay System for Acetyl CoA Carboxylase Activity.......................................122

K. Identification of the Reaction Product........128

L. Biotin Determination...........................128

VII. RESULTS...132

A. Acetyl CoA Carboxylase Activity of Whole Tissue Homogenates..........................132

B. Soluble Leaf Acetyl CoA Carboxylase...........133

C. Chloroplast Acetyl CoA Carboxylase............136

Chlorophyll Content per Plastid..............136

Bacterial Contamination in Plastid Isolations..............................140

Enzyme Activity of Chloroplast stroma Preparations...........................140

Tests to Determine the Presence of an Inhibitor..............................145

D. Assay Constituents...........................147

E. Inhibitory Effect of Avidin..................147

F. End product Analysis.........................147

G. Time Curve...................................148

H. Biotin Content of Chloroplast Fractions.......153

VIII. DISCUSSION....................................155

REFERENCES...166

List of Tables

Table Page

I. Lipid composition of maize and spinach chloroplasts.21

II. Response factors of the flame ionizing detector for fatty acid methyl esters..............................68

III. Gravimetric determination of the total lipid content of barley leaf segments using three extraction procedures...................................77

IV. Gramine content of the apical 3 cm leaf segments of 4 and 7 day old mutant and normal barley seedlings..80

V. Estimation of individual lipid constituents by either phosphorus or galactose analysis and with the C:17 internal standard..........................88

VI. Pigment ratios estimated from sucrose 2D TLC plates of pigment extracts from the apical 3 cm leaf segments of normal and mutant barley seedlings harvested at daily intervals from 4 to 8 days......103

VII. Acetyl CoA carboxylase activity of whole cell homogenates of mutant and normal barley seedlings from 1 to 6 days of age expressed on protein, fresh weight and per seedling basis......................135

VIII. Acetyl CoA carboxylase activity of chloroplast stroma and lamellar fractions isolated from the apical 3 cm leaf segments of 4 and 5 day old normal barley seedlings..................................137

IX. Chlorophyll content per plastid from the apical 3 cm leaf segments of normal and mutant barley seedlings at 4, 6 and 8 days and the percentage chlorophyll in the mutant relative to the normal expressed on a fresh weight and per plastid basis..141

X. Estimation of numbers of bacteria and plastids from chloroplast fractions prepared from 6 and 8 day mutant and normal barley seedlings..................141

XI. Acetyl CoA carboxylase activity of chloroplast stromal preparations from apical 3 cm leaf segments of 4, 6 and 8 day old normal and mutant barley seedlings....................................143

XII. Effect of the addition of *E. coli* carboxyltransferase subunit to a stromal preparation (from 5 day normal barley) on acetyl CoA carboxylase activity..146

XIII. Effect of pooling stromal preparations and whole tissue homogenates of barley seedlings on acetyl CoA carboxylase activity..........................146

XIV. Cofactor requirements and the effect of other additives on acetyl CoA carboxylase activity.......148

XV. Effect of the addition of avidin to stromal preparations of 5 day normal and 6 day mutant and to 2 day normal and mutant whole tissue homogenates on acetyl CoA carboxylase activity.................154

XVI. Biotin content of chloroplast stroma and lamellar fractions from the apical 3 cm leaf segments of 5 day mutant and normal barley seedlings.............154

List of Figures

Figure Page

1. Diagrammatic representation of chloroplast lamellae in a grana stack showing large and small fracture particles..19

2. Chloroplast lamellae membrane model showing the photosystem I and II reaction centres, light harvesting complexes and the proposed fracture line which limites the large freeze fracture particles to the inner membrane leaflet..........................19

3. Absorption scan of the phosphomolybdate complex (A) and a curve used for phosphorus estimations(B).......71

4. Absorption scans of galactose and sugar constituents of monogalactosyl diglyceride (MG), digalactosyl diglyceride (DG) and sulfoquinovosyl diglyceride (SL) from barley leaves estimated by the phenol-H_2SO_4 procedure (A) and a typical curve used for galactose estimations (B)........................71

5. Absorption scan of the ammonium complex estimated by the phenol-hypochlorite procedure (A) and a typical standard curve used for nitrogen estimations (B).....71

6. Absorption scan of the gramine-xanthydrol complex (A) and a representative standard curve used for gramine estimations (B)...........................71

7. Outline of flavonoid-like compounds, present in the chloroform:methanol (2:1, v/v) lipid extract, from 6 day mutant barley leaves separated by one dimensional paper chromatography......................82

8. Outline of flavonoid-like compounds, present in the chloroform:methanol (2:1, v/v) lipid extract from 6 day mutant barley leaves separated by two dimensional paper chromatography......................82

9. Spectral scans of flavonoid-like compounds present in the chloroform:methanol (2:1, v/v) lipid extract from 6 day mutant barley leaves......................82

10. Outline of a representative two-dimensional thin-layer chromatogram of the polar lipids isolated from leaf segments of 4, 6 and 8 day mutant and normel barley seedlings..............................84

11. Infra-red spectra of authentic gramine (A) and gramine isolated from 8 day mutant (B) and 8 day normal (C) barley leaves..............................86

12. Protein and lipid content of the apical 3 cm leaf segments of 4, 6 and 8 day mutant and normal barley seedlings..92

13. Chlorophyll content of the apical 3 cm leaf segments of 4, 6 and 8 day mutant and normal barley seedlings.92

14. Acyl lipid content of the apical 3 cm leaf segments of 4, 6 and 8 day mutant (A) and normal (B) barley seedlings..94

15. Acyl lipid content expressed as a proportion of total acyl lipid content for the apical 3 cm leaf segments of 4, 6 and 8 day old mutant and normal barley seedlings.....................................94

16. Acyl lipid content of the second 3 cm leaf segments of 6 and 8 day old mutant (A) and normal(B) barley seedlings.....................................94

17-23. Changes in the fatty acid composition of acyl lipids from leaf segments of 4, 6 and 8 day old mutant and normal barley seedlings.............................96

24. Outline of a representative two-dimensional thin-layer chromatogram of leaf pigments isolated from the apical 3 cm leaf segments of 4 to 8 day old mutant and normal barley seedlings..................100

25-29. Absorption spectra of chlorophylls and carotenoids isolated from the apical 3 cm leaf segments of 7 day mutant barley seedlings and separated by 2D TLC on powdered sucrose plates...........................100

30. Chlorophyll and carotenoid content of the apical 3 cm leaf segments sampled at daily intervals from 4 to 8 days of normal (A) and mutant (B) barley seedlings..102

31. Brief outline of procedure used for partial purification of acetyl coenzyme A carboxylase subunits from E. coli cells........................124

32. Partial purification of the carboxyltransferase subunit of acetyl CoA carboxylase from E. coli cells..124

33. Standard curve used to determine specific activity of $1\text{-}^{14}C$ sodium bicarbonate solution................127

34. Absorption scans and standard curve of biotin standards quantified with the avidin dye binding method..131

35. Absorption scans and standard curve of biotin standards following protease digest.................131

36. Regression line used to determine the number of plastids from the chlorophyll concentration of an isolated plastid fraction of 6 day normal barley....139

37. Fractionation of the products of the acety CoA carboxylase enzyme assay of 6 day mutant stromal preparations.....................................150

38. Time curve for acetyl CoA carboxylase activity from normal barley....................................152

ABBREVIATIONS

5-ALA	5- (delta) aminolevulinic acid
BC	biotin carboxylase
BCCP	biotin carboxyl carrier protein
BSA	bovine serum albumin
C/M	chloroform/methanol
Chl	chlorophyll
CoA	coenzyme A
CPI	chlorophyll protein complex I
CPII	chlorophyll protein complex II
CT	carboxyltransferase
C 16	palmitic acid
C 17	heptadecanoic acid
C 18:1	oleic acid
C 18:2	linoleic acid
C 18:3	linolenic acid
C 16:1-3t	trans-3-hexadecanoic acid
DPM	disintegrations per min
DTT	dithiothreitol
Ea	BC+BCCP subunits
Eb	CT subunits
HABA	2(4-hydroxyazobenzene) benzoic acid
LHC a/b	light harvesting chlorophyll a/b
LS	large subunit
DG	digalactosyl diglyceride
MG	monogalactosyl diglyceride
PE	phosphatidyl ethanolamine
PG	phosphatidyl glycerol
PC	phosphatidyl choline
Pchlide	protochlorophyllide
PLB	prolamellar body
PSI	photosystem I
PSII	photosystem II
RUBP	ribulose bisphosphate carboxylase
SL	sulfolipid
SDS	sodium dodecyl sulfate
SDBS	sodium dodecylbenzene sulfate
TLC	thin layer chromatography
2-D TLC	two-dimensional chromatography

I. INTRODUCTION

Studies on a virescens mutant of Gateway barley have been pursued in this laboratory for a number of years. The mutation was classified as the virescens type according to the scheme outlined by Gustafsson, Walles and von Wettstein (1962) as reviewed by Walles (1971). This scheme is based on the chloroplast phenotype in which the symptoms are a reduced amount of chlorophyll. The virescens mutants differ from the more frequent 'static' mutations in that the genetic lesion appears to be self-correcting since the leaves which are initially chlorophyll deficient slowly turn green as the leaf develops, although they are still somewhat chlorophyll deficient at maturity. Leaf ultrastructural studies of Gateway barley and its virescens mutant reveal a general time lag in the mutant in the development of the chloroplast internal membrane system (Maclachlan and Zalik, 1963; Jhamb and Zalik, 1975). The incorporation of U-^{14}C leucine into a leaf protein fraction was less efficient in the mutant at an early growth stage suggesting a lower rate of protein synthesis (Sane and Zalik, 1970). Also plastids from young mutant leaves were found to be deficient in some membrane protein bands which were present in the normal of corresponding age. However as development progressed the protein gel patterns of both plant types became similar (Jhamb and Zalik, 1973). The photoreductive activities of the mutant plastids were also initially very low but reached normal levels which corresponded with an increase in the chlorophyll content and the formation of well developed grana (Horak and Zalik, 1975). The initial pigment deficiency of the virescens barley is suggested not to result from a blockage in the biosynthetic pathway of chlorophyll since the addition of 5-aminolevulinic acid increased the amount of chlorophyll to the same extent in both the normal and mutant (Sane and Zalik, 1970). Low chlorophyll content which is not due to a blockage in chlorophyll synthesis is common to many pigment deficient mutants. The deficiency is suggested to be one of several pleiotropic respones resulting from the failure to make a specific gene product that is required for normal chloroplast formation (Gillham _et al_., 1978). Even though the study of the primary lesion is complicated by the pleiotropic effect, the virescens mutant is of interest in structural-functional analysis and developmental studies of the chloroplast and provides an additional system to the frequently used greening of etiolated seedlings, growth under intermittent light and membrane fractionation studies.

The virescens mutation was shown to be due to a single recessive gene which gives a Mendelian pattern of inheritance, suggesting the gene affected was located on chromosomes in the nucleus (Walker _et al_., 1963; Stephansen and Zalik, 1971). Although plastids contain their own DNA and protein synthesizing system, several genes concerned with their development are inherited in a Mendelian fashion and many chloroplast proteins are found to be synthesized on cytoplasmic ribosomes (Ellis, 1977; Kung, 1977; Gillham *et al.*, 1978; Yoshida, 1979). It therefore appears the formation of the chloroplast involves a complex integration of the expression of genetic information present in both the plastid and nuclear genome. This mutant is thus also of interest in the study of intergenome cooperation during chloroplast formation.

The literature review will therefore cover studies pertaining to the structure and function of the chloroplast internal membrane system including both membrane fractionation techniques and developmental studies. Reference will also be made to studies dealing with the cellular location and origin of the proteins involved in membrane synthesis. These will include the biosynthesis of thylakoid proteins and enzymes associated with the biosynthesis of pigments and lipids. The review will deal primarily with studies on higher plants, also the areas pertaining to the lipid composition and acetyl CoA carboxylase enzyme will be covered in more detail.

Since the chloroplast plays a very active role in cellular lipid metabolism and displays an unique acyl lipid content it was of interest to study the lipid content and acetyl CoA carboxylase activity to further characterize the virescens mutant.

Thus the research part of the thesis is divided into two sections, the first dealing with the acyl lipid and pigment compositional changes associated with the development of the barley seedlings. The second section is a study of acetyl CoA carboxylase activity in developing barley seedlings and isolated plastids.

II. LITERATURE REVIEW

A. Overall Structure, Activity and Composition of the Chloroplast

Internal Membrane Network

The mature chloroplast in higher plants appears lens shaped with an average diameter of about 5 um (Kirk, 1967; Bogorad, 1975; Larsson *et al.*, 1977). Electron microscopic observations of thin sections revealed the plastid is enclosed by two envelope membranes separated by a space of approximately 20 nm (Boardman *et al.*, 1978) and contains an internal membrane network that appears to be portions of flattened vesicles or thylakoid seen in cross section. In several locations within the thin section many thylakoids are stacked upon one another and these regions are joined by single unstacked thylakoids (Kirk, 1967; Park and Sane, 1971). The stacked regions are termed grana whereas the unstacked regions are referred to as the stroma lamellae. These terms are suggested to have arisen from early light microscopic observations in which small dots or granules were observed within the mature plastid (Granick and Porter, 1947; Muhlethaler, 1971; Bogorad, 1975). The chloroplast internal membrane system is often referred to as vesicles, thylakoids or lamellae. Several workers have attempted to define the three dimensional arrangement of the stroma and grana lamellae (Park and Sane, 1971). A recent model, based primarily on electron microscopic observations of thin sections, suggests that the internal membrane network constitutes a continuous membrane enclosed cavity (Park and Sane, 1971; Boardman _et al_., 1978).

Plastoglobules

Plastoglobules are also a characteristic chloroplast constituent. They are often referred to as osmiophilic globules due to their strong staining reactions with osmium tetroxide. They occur within the stroma matrix and can be isolated free from the lamellar network by mild sonication (Greenwood _et al_., 1963; Barr _et al_., 1967) or by needle valve disintegration (Bailey and Whyborn, 1963) followed by high speed centrifugation. Their lipoidal content makes them less dense than the lamellar network. Isolates from developing leaves were found, using silica gel chromatography, to contain high amounts of chloroplast quinones such as; plastoquinone, α-tocopherol, α-tocoquinone and phylloquinone and to lack carotenoids and chlorophyll (Bailey and Whyborn, 1963; Greenwood _et al_., 1963; Barr _et al_., 1967; Lichtenthaler, 1969; Lichtenthaler, 1969b). The number and size of plastoglobules increases with the age of the leaf and in senescing tissue the degenerating chloroplasts have predominantly large plastoglobules (Lichtenthaler, 1969b; Freeman _et al_, 1978), which contain carotenoids in addition to the plastid quinones (Lichtenthaler, 1969b).

Photochemical Activity

Studies during the 1950's (reviewed by Arnon, 1977) established that the isolated chloroplasts were capable of complete photosynthesis, that is the photoassimilation of CO_2. Trebst *et al*. (1958) physically separated the 'light' and 'dark' reactions by high speed centrifugation of osmotically disrupted chloroplasts. The pelleted membrane system, which contained all the chlorophyll, had the potential to form 'assimilatory power' upon illumination and when added to the soluble stroma fraction was able to resume the CO_2 assimilation reactions. During the early 1960's the energy rich products essential for the conversion of CO_2 into organic compounds were identified as NADP, ATP and reduced ferredoxin (Arnon, 1977). Thus the enzyme systems for the light reactions providing the photoreductive and photophosphorylation activity are membrane bound while those associated with CO_2 fixation are present in the soluble fraction.

Chemical Composition

The complete chemical composition of the chloroplast is difficult to estimate due to the ease with which the water soluble proteins are lost during isolation. Kirk (1967) has applied the ratio of soluble to insoluble protein obtained from chloroplasts isolated by non-aqueous techniques, which are presumed to retain much of their soluble protein, to make corrections to published data. On percentage dry weight spinach chloroplasts contained 69% protein of which 31% was membrane bound and the total lipid content was estimated at 21% (Kirk, 1967). On the other hand cauliflower leaf chloroplasts were reported to have a total lipid content which represented 34% of the lipid plus protein content on a weight comparison basis (Schwertner and Biale, 1973).

The chloroplast fraction from spinach leaves was estimated, on a chlorophyll basis, to contribute two-thirds of the total leaf lipid content (Zill and Harmon, 1962). Generally this fraction showed a less complex lipid pattern than that of the whole leaf tissue (Allen et al., 1966) and for spinach it lacked two neutral lipids normally present in the whole leaf extract which were tentatively identified as cuticular waxes and long chain alcohols (Zill and Harmon, 1962). Lipids make up approximately 7-10% of the dry weight of green leaves in higher plants (Kates, 1970; Hitchcock and Nichols, 1971). Estimates on the contribution of the chloroplast fraction to the total leaf protein are available from the work of Zucker and Stinson (1962) on mature Oenothera leaves where approximately 75% of the leaf protein was contributed by the chloroplast fraction. Comparisons for this study were also standardized to chlorophyll content and the ratios of soluble to insoluble protein were similar to those used by Kirk suggesting minimal loss of stromal proteins. It is of interest that the major plant enzyme ribulose bisphosphate carboxylase which occurs in the soluble fraction of the chloroplast contributes up to 50% of the total soluble leaf protein (Ellis, 1976).

B. Chloroplast Membrane Studies

Separation of Photosystems

Following the discovery that the photochemical activity and chlorophylls were located exclusively in the lamellar system attempts were made to obtain the complete chemical composition and structural assembly of the internal membrane network (Lichtenthaler and Park, 1963). Attempts were also made to fractionate the lamellae into the smallest unit (quantosome) which could carry out the photochemical activity (Park and Pon, 1963). However, it was soon established that the photosynthetic electron transport system consisted of two photosystems (Clayton, 1963; Boardman, 1970; Arnon, 1971; Sane and Park, 1971) and the photosystems could be separated into active photosystems I and photosystem II (PSI, PSII) enriched fractions by treatment of the membranes with nonionic detergents (Anderson and Boardman, 1966; Vernon et al, 1969) or by mechanical shearing (Sane and Park, 1971) indicating that differentiation of activity must exist along the lamellar membrane. Questions were raised as to how the lamellar network was fragmented and on what basis should compositional results be expressed. Park and Sane (1971) suggest the photosystems are integral to the lamellar system and that both detergent treatment and shearing fragment the lamellae into stroma and grana regions. They point out that separation of the photosystems does not occur in low ionic buffers in which grana unstacking takes place (Izawa and

Good, 1966). Arntzen *et al.* (1969) initially suggested digitonin splits the lamellar bilayer into inner and outer halves, with the inner leaflet half containing the large freeze fracture particles and enriched PSII activity, while the outer leaflet half contained the small freeze fracture particles and PSI activity. It was later established however that the fragmentation pattern was very dependent on the experimental system and the detergent concentration used. The grana fraction obtained by mechanical shearing could be further fragmented into PSI and PSII enriched fractions using detergents (Jacobi, 1977; Wessels, 1977).

Lipid Composition of Stroma and Grana Membrane Fragments

Although there is now agreement that the chloroplast lamellae could be fragmented into stroma and grana fractions with low concentrations of nonionic detergents or mechanical shearing the quantitative recovery of the separated fractions differed. This was particularly evident with detergent fragmentation studies where the concentration of detergents and exposure time as well as the ionic strength of the fragmentation media differed among laboratories (Jacobi, 1977). These differences were suggested to be primarily due to the additional fragmentation of the grana and the modifying effect of the detergents on the membrane lipid constituents, causing the solubilized lipids to be released and/or exchanged between the fragments (Park and Sane, 1971; Jacobi, 1977). In general the stromal fractions enriched in PSI activity, obtained with Triton X-100 or digitonin (Huzisige _et al._, 1969; Vernon _et al._, 1969) or by mild sonication (Wintermans, 1971), were found to be higher in galactolipids, cytochrome f and b_6 and plastoquinones than the grana fragments enriched in PSII, when results were expressed on chlorophyll content. Chloroplasts from the bundle sheath cells of maize and sorghum which have very limited grana stacking also showed a significantly higher galactolipid content on a chlorophyll basis than the mesophyll chloroplasts, from the same plants, which contained good grana stacks (Bishop, 1971).

Allen _et al._ (1972) suggest, due to the low chlorophyll content of the stroma lamellae, chemical composition standardized to chlorophyll alone could be misleading. They report that the stroma and grana enriched lamellar fragments prepared by the French press technique and washed to remove extrinsic proteins showed similar relative amounts of the major chloroplast glycerol lipids: monogalactosyl diglyceride (MG), digalactosyl diglyceride (DG), phosphatidyl glycerol (PG) and sulfolipid (SL) when results were expressed on protein content. The grana fraction however contained 1.5 times more chlorophyll when expressed on a protein basis. Further study on the carotenoid composition indicated the β-carotene content normalized to protein content was also similar for both fractions but the grana fraction contained considerably more xanthophylls (Trosper and Allen, 1973). The carotenoid to chlorophyll ratios however were similar for both fractions (Trosper and

Allen, 1973).

Subfractionation of the Photosystem Complexes

Further fragmentation and purification of the stroma and grana membrane fractions has resulted in the isolation of a large number of particles. Jacobi (1977) outlined a five-category classification scheme to describe these subchloroplast particles which range from large complexes which retain electron transport as well as photophosphorylation activity, to the very small particles with limited activity. Five distinct enzyme complexes have been partially purified from the lamellar membranes which either retain the activity of the individual partial reactions or retain the ability to reconstitute with other membrane constituents to form active complexes (Arntzen, 1978). As previously noted, the grana lamellae can be further fragmented by additional detergent treatment into PSI and PSII enriched fractions. The stroma PSI and grana PSI are found to be essentially identical in polypeptide composition and electron transport activity (Nolans and Park, 1975; Wessels and Borchert, 1975). The PSI and PSII enriched fractions have been further subfragmented using a combination of nonionic detergents, hydroxylapatite chromatography and sucrose density gradient procedures. The PSI particles are further separable into PSI reaction center (P-700 chlorophyll a complex) and a cytochrome f-b_6 complex (Thornber, 1975; Wessels and Borchert, 1975). Chlorophyll a and β-carotene accounted for essentially all the pigment in the P-700 Chl a protein complex with a molar ratio of Chl a/β-carotene of 20-30/1. Chromatography of the chlorophyll extract revealed the absence of chlorophyll b (Thornber, 1975). Several polypeptides, primarily in the 10 to 74 kilo-dalton (KD) range were associated with the lipid extracted SDS or SDBS solubilized reaction center (Wessels and Borchert, 1975).

The PSII enriched particles were further separated into a PSII reaction center and a light harvesting chlorophyll a/b (LHC a/b) protein complex (Arntzen, 1978; Boardman _et al_., 1978). Essentially all the pigment of the reaction center was chlorophyll a (Chl a/b 25-28/1) and low amounts of β-carotene and lutein were present (Thornber, 1975; Boardman _et al_., 1978). At least 6 polypeptides in the 27 to 54 KD range were detected (Arntzen, 1978; Thornber, 1975; Boardmen _et al_., 1978). The LHC a/b protein complex is photochemically inactive. It has a Chl a/b ratio of approximately 1 and contains all 4 carotenoids found in the chloroplast with lutein and β-carotene as the major carotenoids (Thornber, 1975). The chlorophyll to carotenoid ratio varies from 3-7/1 (Thornber, 1975). Several laboratories have provided evidence that the complex contains at least two polypeptides in the 23-27 KD range (Wessels and Borchert, 1975; Arntzen, 1978; Boardman _et al_., 1978).

An estimate of the quantity of pigment and protein in the respective photosystem fragments is available from the early work of Ogawa _et al_. (1966) and Thornber _et al_.

(1967). By completely solubilizing the whole lamellar membrane with ionic detergents (SDS or SDBS) followed by electrophoretic fractionation on SDS-polyacrylamide gels three pigment zones were separated. These were termed components I, II and III corresponding to their increasing electrophoretic mobility. Components I and II have been inferred by Thornber et al. (1967) and several workers (cited by Thornber, 1975) studying membrane fragments enriched in the respective photosystems to be the pigment complexes of PSI and PSII respectively and are frequently referred to as chlorophyll protein complex I and II (CPI, CPII). Component III represented a free pigment detergent complex. CPI and II respectively accounted for 20% and 50-60% of the chlorophyll in the initial extract (Ogawa et al., 1966) and 28% and 49% of the protein (Thornber et al., 1967). CPII was later inferred to be the LHC a/b protein (Thornber, 1975; Boardman et al., 1978). Very recently several laboratories (cited by Thornber et al., 1979) using improved extraction techniques and electrophoresis systems reported that virtually all the chlorophyll in the lamellar membrane is complexed with protein. Several new chlorophyll protein complexes have been separated and are suggested to represent reaction center chlorophyll proteins as well as oligomers of the LHC a/b complex and CPI complex (Anderson et al., 1978; Henriques and Park, 1978; Markwell et al., 1979; Thornber et al., 1979). Boardman et al. (1978) and Thornber et al. (1979) outline some of the difficulties associated with attempts to identify the chlorophyll-protein complexes since the association of the non-covalently bound lipophilic pigments with proteins in the presence of strong ionic detergents like SDS is at present not understood. The complexes are assumed to retain at least part of their tertiary structure during fractionation.

C. Chloroplast Membrane Model

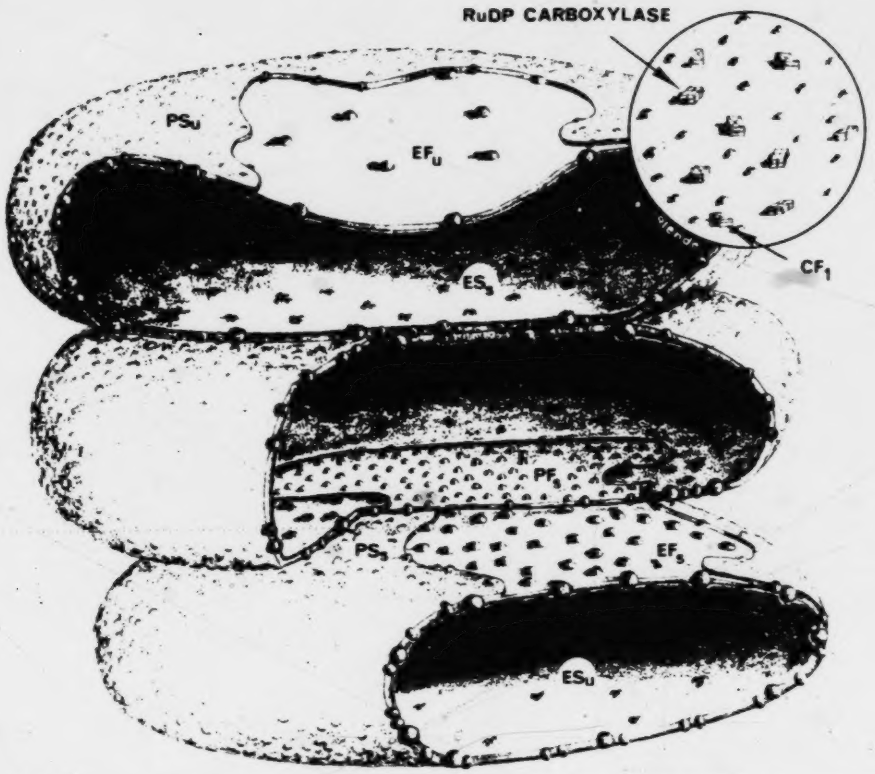
Freeze Fracture Pattern and Chlorophyll Protein Complexes

Several membrane models have been proposed which attempt to outline the structural arrangement of the photosystems, the chlorophyll-protein complexes and the ATPase complex. Kirk (1971) and Muhlethaler (1977) reviewed evidence from earlier membrane models and suggested the chloroplast data best fit the lipid matrix model. Anderson (1975) extended the fluid-lipid protein mosaic model (Singer and Nicolson, 1972) to include the unique features of the chloroplast lamellar membrane. The uniqueness of the membrane is due to its differentiation into grana and stroma regions and also due to the freeze fracture pattern of the grana lamellae. The fracture technique which cleaves the bilayer membrane along its inner hydrophobic region (Branton, 1966) displays distinct large particles integral to the inner membrane leaflet and smaller particles within the outer membrane leaflet. These inner and outer leaflet fracture planes are refered to as the exoplasmic and protoplasmic fracture surface respectively using the terminology of Branton _et al_. (1975). A diagrammatic representation of stacked lamellae containing the freeze fracture particles as outlined by Arntzen (1978) is shown in Figure 1. Evidence presented in the recent reviews indicates the presence or absence of the large freeze particles corresponds to the presence or absence of the LHC a/b protein complex and these large freeze fracture particles are mainly confined to the grana (Anderson, 1975; Arntzen, 1978; Boardman _et al_., 1978). Good PSII activity however occurs in chloroplasts where grana stacking does not occur as in some algae or in higher plants affected by mutation or grown under restrictive light regimes (Anderson, 1975) suggesting neither grana stacks nor large freeze fracture particles are required. However in the fully mature chloroplasts with well formed grana, PSII activity is mainly confined to the grana region (Park and Sane, 1971; Jacobi, 1977). Thus the study of mature grana containing chloroplasts has led to the generally accepted view that a differentiation of the photochemical activity occurs between the stacked and unstacked regions. Boardman _et al_. (1978) suggest that if PSII is confined to the grana region then the LHC a/b protein complex could be responsible for this segregation. Since the LHC a/b protein complex accounts for approximately 50% of the intrinsic protein, Anderson (1975) suggests the large fracture particles contain the LHC a/b protein complex and the small fracture particles contain CPI since PSI enriched membrane fragments contain only the small particles. A diagram of a model recently proposed by Arntzen (1978) is shown in Figure 2. The large freeze fracture particles are outlined as a PSII reaction center surrounded by a discrete LHC a/b protein complex. The separability of the center from the light harvesting complex is consistent with the subfractionation studies and the observed PSII activity in agranal chloroplasts lacking the large fracture particles (Anderson, 1975) as well as the higher light intensities required to saturate PSII activity from agranal chloroplasts deficient in the LHC a/b protein complex (Arntzen _et al_., 1977). In addition the discrete stepwise increase in the particle size during development suggests the accumulation of aggregates of the LHC a/b protein complex presumably localized around the PSII center (Arntzen _et al_., 1977). The inclusion of the cytochrome complex and hydrophobic protein moiety of the coupling factor in the small fracture particles of this model was according to Arntzen (1978) largely based on conjectural evidence. The integral placement within the membrane and the protein nature of the fracture particles is further supported by studies with protease enzymes which reduced the size of the particles (Bamberger and Park, 1966; Machold _et al_., 1977). The arrangement of the electron transport chain constituents with respect to the bulky chlorophyll protein complexes is not known and Anderson (1975) suggests their relatively small mass would make identification of their polypeptides

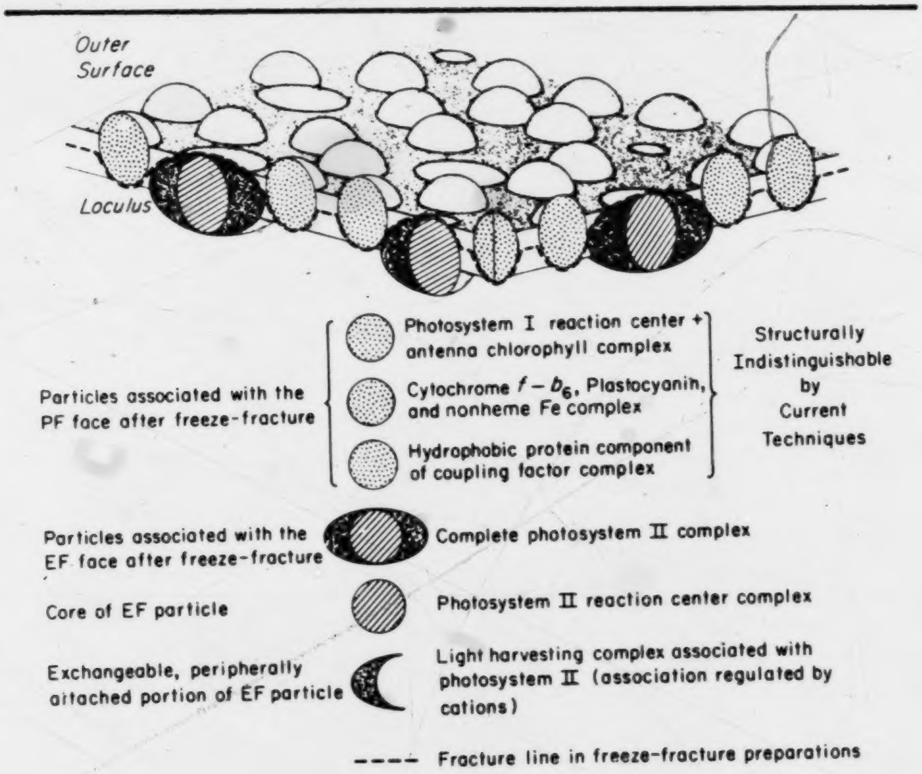
Figure 1. Diagrammatic representation of chloroplast lamellae in a grana stack showing large and small freeze fracture particles. EF and PF refer to exoplasmic and protoplasmic fracture surfaces respectively. Subscripts s and u refer to stacked and unstacked membrane regions respectively (From Arntzen, 1978).

Figure 2. Chloroplast lamellae membrane model showing the photosystem I and II reaction centers, light harvesting complexes and the proposed fracture line which limits the large freeze fracture particles to the inner membrane leaflet (From Arntzen, 1978).

1


RuDP CARBOXYLASE
CF₁
PSu
EFu
ESs
PFs
PSs
EFs
ESu

2


Outer Surface
Loculus
Photosystem I reaction center + antenna chlorophyll complex
Cytochrome f – b6, Plastocyanin, and nonheme Fe complex
Hydrophobic protein component of coupling factor complex
Particles associated with the PF face after freeze-fracture
Structurally Indistinguishable by Current Techniques
Particles associated with the EF face after freeze-fracture
Complete photosystem II complex
Core of EF particle
Photosystem II reaction center complex
Exchangeable, peripherally attached portion of EF particle
Light harvesting complex associated with photosystem II (association regulated by cations)
Fracture line in freeze-fracture preparations

difficult.

The fluidity of the membrane is suggested by the unstacking and restacking studies. Grana unstack when suspended in low ionic buffers and restack when transferred to buffers of higher ionic concentration. During the unstacking and restacking process the conservation of the fracture particles size and number suggests lateral movement of the particles within the plane of the membrane (Ojakian and Satir, 1974).

Acyl Lipids as Matrix and Boundary Lipids

Lipids are estimated to make up 50% of the spinach thylakoid membrane content (Lichtenthaler and Park, 1963; Allen et al., 1966). Some estimates are available on the lipid classes present in the total lipid extract and are shown in Table I. The major acyl lipids MG, DG, PG and SL account for approximately 50% of the total chloroplast lipid content (Table I, Leech and Murphy, 1976; Mazliak, 1977). The galactolipids MG and DG are by far the most predominant accounting for 40 to 45% of the total lipids in spinach, maize and bean chloroplasts (Table I; Leech and Murphy, 1976). PG was shown to be the major phospholipid representing 60-65% of the phospholipid content of spinach, bean and tobacco chloroplasts (Ongun et al., 1968; Kates and Marshall, 1975) however, the total phospholipids accounted for only 10% of the chloroplast lipids (Table I; Leech and Murphy, 1976). Sulfolipid (SL) accounted for approximately 5% of the chloroplast membrane lipid (Table I; Leech and

Table I. Lipid composition of maize and spinach chloroplasts.

	grams per 100 grams lipid	
	Spinach[1]	Maize[2]
Chlorophyll	20.8	13.6
Carotenoids	2.8	3.7
Quinones	3.3	1.0
Phospholipids	9.1	5.5
Galactolipids	40.2	47.4
Sulfolipid	4.1	4.5
Steroids	2.2	

[1] Kirk, 1967.

[2] Mudd and Garcia, 1975.

Murphy, 1976), and although present in minor amounts in non photosynthetic cells it is concentrated in the plastid fraction of photosynthetic tissue (Ongun _et al_., 1968; Benson, 1971; Mudd and Garcia, 1975; Harwood and Nichols, 1979). Chloroplasts of higher plants also contain very high levels of polyunsaturated fatty acids (Kates, 1970; Hitchcock and Nichols, 1971) concentrated primarily in the acyl moieties of the major galactolipids (Mudd and Garcia, 1975; Leech and Murphy, 1976; Mazliak, 1977). Also unique to the chloroplast is the fatty acid trans-3-hexadecanoic acid (16:1-3t) which is found specifically in photosynthetic tissue (Leech and Murphy, 1976) and esterified primarily to PG (Harverkate and Van Deenen, 1965; Allen _et al_., 1966; James and Nichols, 1966; Harwood and James, 1975). The very high galactolipid content, restricted phospholipid composition and fatty acid content differ considerably from the lipid composition of other cellular membranes;

mitochondria, peroxisomes and microsomes (Allen et al., 1966; Leech and Murphy, 1976; Mazliak, 1977). This unique acyl lipid composition is ubiquitous in higher plants (Harwood and Stumpf, 1976). Harwood and Stumpf (1976) suggest this indicates they likely play a vital role in photosynthesis.

Several studies have attempted to relate the specific lipid composition to photosynthetic functions. These have included the effect of lipid removal, either by lipases or organic solvents, on structural characteristics and enzyme activities. Treatment of chloroplast membrane fragments with a crude galactolipase preparation destroyed the normal freeze fracture pattern suggesting galactolipids are the major constituents of the membrane matrix (Bamberger and Park, 1966). Differential lipid extraction with increasing acetone concentration (Swanson et al., 1973) or complete lipid removal with chloroform/methanol (Yao et al., 1972) disrupted the electron microscope membrane images of the chloroplast lamellae of both unfixed and tissue previously fixed with glutaraldehyde, also indicating the lipid involvement in membrane assembly.

A requirement of specific lipids, if any, for the chloroplast membrane enzyme activity has not been demonstrated. In general mild lipid extractions result in little membrane disturbances and full activity is usually restored by addition of the easily solubilized electron transport chain constituents such as plastoquinones (Amez,

1977) and plastocyanins (Krupa and Basynski, 1975; Katoh, 1977). The fluid mosaic model (Singer and Nicolson, 1972) proposes that wh of the membrane lipids exist in the bilayer formation and are not strongly associated with the integral proteins, a minor lipid fraction coupled with the integral proteins might be specific for the function of the proteins. The results of Shaw et al. (1976) could perhaps be explained on this basis, where removal of 70% of MG and 50% of the DG content from the chloroplast membranes with a galactolipase preparation had no adverse effect on the electron flow through PSI and PSII provided that bovine serum albumin (BSA) was present to bind the released fatty acids. Also chloroplast lamellae treated with snake venom phospholipase A_2 which removed over 80% of the phospholipids retained 80% of their electron transport activity and 64% of their initial cyclic photophosphorylation activity in the presence of BSA (Hirayama and Nomotobori, 1978). Hirayama and Nomotobori (1978) suggest their results indicate the phospholipids of the chloroplast lamellae are present in two states with the 20% not attacked by the phospholipase representing the boundary lipid fraction which has a role in the photoactivities. Anderson (1975) suggests SL, PI and PG due to their anionic nature, could serve as boundary lipids for the large freeze fracture particles and take part in membrane stacking since cations are required to preserve grana stacks or to initiate restacking. Some authors have suggested reconstitution studies would be a useful approach to study the relationship of specific lipids to the photoactivities (Harwood and Stumpf, 1976; Leech and Murphy, 1976). However complete removal of lipids from membrane associated enzymes is difficult to achieve with detergents (Sandermann, 1978) and near complete removal with organic solvents results in an irreversible aggregation and denaturation of many membrane proteins (Helenius and Simons, 1975). Also most reconstitution studies have shown that a mixture of polar lipids best restores the enzyme activity (Sandermann, 1978).

D. Chloroplast Development

Plastid Types

The chloroplast develops from small proplastids, ranging in diameter from 0.5 to 1 um, present in meristematic cells (von Wettstein, 1958; Leech et al., 1973; Robertson and Laetsch, 1974). The number of plastids per cell is also known to increase during tissue maturation. Dividing plastids are difficult to detect in meristematic tissue since they are not easily distinguishable from mitochondria (Stubbe, 1971) however, the increase in the number of plastids per cell from approximately 30 to 70 in developing barley leaves (Robertson and Laetsch, 1974) and the observation of fission configurations (Kirk, 1967; Stubbe, 1971) suggest division occurs during leaf growth and can occur beyond the proplastid stage. A close positive correlation was shown to exist between the number of plastids in the cell and the amount of DNA in the nucleus (Butterfass, 1973). At least 4 plastid type phenotypes are distinguishable in higher plants, these include the amyloplast, etioplast, chloroplast and chromoplast (Kirk, 1967; Muhlethaler, 1971; Stubbe, 1971). Intermediate types are common and it was suggested they are all initially derived from the meristematic proplastid (Muhlethaler, 1971). The amyloplasts are mainly found in storage tissue such as cotyledons, endosperm and tubers (Kirk, 1967), while the chromoplasts occur in special organs such as petals, fruits and some roots (Kirk, 1967). Etioplasts represent an arrested stage of chloroplast development in angiosperms grown in the dark. Gymnosperms and several algae are able to form chlorophyll and elaborate lamellar structure in the dark (Wettstein, 1958; Kirk, 1967; Ohad, 1975; Bogorad, 1976) however, angiosperms do not form chlorophyll but accumulate low levels of protochlorophyllide. The plastids of the angiosperms develop beyond the proplastid stage forming what Kirk (1967) has termed etioplasts. They approach 2/3 the size of the fully developed chloroplast and form e condensed crystalline membrane network termed prolamellar body (PLB) rather than the normal lamellar system (Kirk, 1967). Upon illumination the etioplast is rapidly transformed into fully developed and functional chloroplasts (Robertson and Laetsch, 1974).

Modifications of the typical mature chloroplast ultrastructure are evident in different plant types. Certain plants with the C_4 pathway for CO_2 fixation such as maize and sugarcane contain chloroplasts of two morphological types. Those in the mesophyll cells display the typical well developed grana systems, however, the bundle sheath chloroplasts either lack grana or have very limited lamellae stacking (Laetsch, 1974). Shade plant chloroplasts display very large grana containing as many as 100 thylakoids per granum and the lamellar network displays an irregular arrangement unlike the orientation in one plane as seen in the conventional chloroplasts structure (Boardman, 1977a).

Membrane Differentiation

In addition to the study of membrane fragments, the sequence of biochemical and structural changes during the maturation of the chloroplast has been extensively studied in attempts to correlate composition, activities and structural assembly. The frequently used systems include greening of etiolated tissue, growth under restrictive light intensities or specific wavelength, the analysis of serial sections of monocotyledon leaves in which the cells display a linear array of maturity in the growing leaf, and the use of deficient mutants. Inhibitors selective for the transcription and translation of nuclear and plastid gene products have also found considerable use in algal systems (Ohad, 1975) and isolated plastid preparations (Ellis, 1977). The greening of isolated etioplasts in culture has found limited use since development does not proceed beyond the loose dispersal of the PLB and the bithylakoid stage

(Wellburn and Wellburn, 1972; Rebeiz et al., 1973; Leech, 1976).

The ultrastructural changes during the greening of the etioplast are well documented. The PLB rapidly loses its regularity and is dispersed into perforated membrane sheets which give rise to the primary lamellae during the first 2 hours. The primary lamellae subsequently grows and differentiates into stroma and grana regions which are detected from 2 to 24 h depending on the physiological age of the etioplasts (Kirk, 1967; Henningsen, 1974; Robertson and Laetsch, 1974). The ultrastructural studies show a continuum of membrane structures during the early stages suggesting the PLB supplies the building materials for the primary lamellae formation. Correlated with the membrane assembly are the synthesis of lipids, pigments and proteins and development of photochemical function (Leech, 1976).

All the major acyl lipids of the mature chloroplast are present in the etioplast (Bahl, 1977; Mackender, 1978). Little change in acyl lipid composition occurs during the dispersal of the PLB and primary lamellae formation suggesting the reorganization of existing lipids (Roughan and Boardman, 1972; Sellden and Selstom, 1976). However a notable increase in the acyl lipid content coincides with the grana formation. The increase was mainly associated with galactolipid, MG and DG, displaying up to a 2 fold increase (Tremoliers and Lepage, 1971; Roughan and Boardman, 1972; Sellden and Selstam, 1976). An interesting feature was the occurrence of trans-3-hexadecanoic acid (16:1-3t) associated specifically with PG. The fatty acid was either absent from etiolated tissue (Roughan and Boardman, 1972; Harwood and James, 1975; Mackender, 1978) or present in trace amounts (Tremolieres and Lepage, 1971; Bahl _et al_., 1976; Sellden and Selstam, 1976; Bahl, 1977). Large increases in 16:1-3t representing 20 to 30% of the acyl content of PG were reported (Roughan and Boardman, 1972; Sellden and Selstam, 1976). These increases were most evident during the latter stages of greening which would suggest the acid has a role either in photochemical activity or in grana formation. In agreement with its role in grana formation the concentration of 16:1-3t was higher in the grana lamellae preparations from maize mesophyll chloroplast than in the corresponding stroma lamellae preparations or in the agranal chloroplasts of the maize bundle sheath cells (Tuquet _et al_., 1977). However the exact role of this unique fatty acid is not known. Since it is not present in some blue green algae (James and Nichols, 1966) and photochemical activity occurs in greening bean leaves when only a trace of the acid is present (Roughan and Boardman, 1972) it appears not essential for the photochemical activities. Its occurrence at equal content in a chlorophyll b less barley mutant which diplays limited grana formation relative to that of the normal (Bolton _et al_., 1978) and the formation of good grana stacks while the fatty acid is only present in trace amounts (Roughan and Boardman, 1972) would also tend to negate an essential role in grana formation.

The initial light stimulated reorganization of the etioplast membranes is accompanied by changes in the *in vivo* spectroscopic forms of chlorophyll (Shibata, 1957; Boardman, 1977) which suggest a relationship between the transformation and dispersal of the PLB, the conversion of protochlorophyllide (Pchlide) to chlorophyllide and phytylation to chlorophyll a and the spectral shifts, however the exact sequence of events is not agreed upon (Boardman, 1977; Boardman *et al*., 1978). Illumination of the etioplasts rapidly converts the Pchlide to chlorophyll. This is followed by a lag in additional chlorophyll accumulation which varies from a few minutes to several hours depending on the species and the age of the seedling. The lag is followed by a period of slow accumulation of chlorophyll, about 2.5 h for barley (Boardman, 1977) and a period of rapid chlorophyll accumulation which corresponds to the development of grana lamellae. Chlorophyll b is not initially detectable but is present at very low levels during the lag phase. The chlorophyll b content increases very rapidly during the period of maximum chlorophyll accumulation resulting in a Chl a/b ratio of 3/1. The Pchlide of dark grown bean and barley leaves can be extracted as a pigment protein complex (Pchlide holochrome complex) which retains the ability to be photoconverted to chlorophyllide-a (Boardman *et al*., 1978). The newly formed chlorophyllide-a protein complex of barley is suggested to dissociate into a colorless "photoenzyme" and a chlorophyllide carrier protein (Boardman et al., 1978). The chlorophyll protein complexes are obviously not present in the etioplast membrane however there is not agreement on which polypeptides are present or absent from the etioplast and which are synthesized during greening (Boardman et al., 1978). Some workers claim that all or most of the polypeptides including those of the chlorophyll-protein complexes are present in the etioplasts with some showing selective increases during greening while others claim many of the membrane polypeptides are synthesized during greening.

The development of the photochemical activities of the greening etiolated system is generally consistent with the theory that light triggers the rapid synthesis of reaction center chlorophyll followed by the synthesis of the bulk of the chlorophyll which serves in a light harvesting capacity. This is most evident in the initial increase in the photoactivity rate per unit chlorophyll and the high light intensities required for saturation followed by a decrease in the light saturation point and activity on a chlorophyll basis (Plesnicar and Bendall, 1973; Boardman, 1977). A sequential development of the photosystems has been found by most authors with PSI and its associated electron transport and photophosphorylation activities occurring before those of PSII (Plesnicar and Bendall, 1973; Bendall, 1977; Boardman, 1977). The absence of light does not affect all biosynthetic processes since the etioplasts contain the constituents of the electron transport chain including cytochrome f, b6 and plastocyanin (Plesnicar and Bendall, 1973) as well as the coupling factor for photophosphorylation (Horak and Hill, 1972; Bogorad, 1975). The rapid development of PSI activities suggest chlorophyll is the limiting factor, however the lag in detection of full PSII activities (2.5 h for 8 day barley) suggests the rate limiting factor could be a number of constituents including the water-oxidizing enzyme (Plesnicar and Bendall, 1973). Bogorad (1975) and coworkers showed the osmotic responsiveness and acid/base phosphorylation of lamellar preparations was not correlated with chlorophyll accumulation and also suggested constituents other than chlorophyll are limiting.

During greening of etiolated seedlings under intermittent light the synthesis of chlorophyll b and the LHC a/b protein complex does not occur and long parallel lamellae develop with little overlap (Sironval _et al._, 1969; Argyroudi-Akoyunoglou _et al._, 1972). The plastids are active in whole electron transport from water (Arntzen _et al._, 1977). When exposed to continuous light extensive membrane growth occurs accompanied by grana formation, a decline in the Chl a/b ratio, the appearance of the LHC a/b protein complex and a stepwise increase in the large freeze fracture particle size (Arntzen _et al._, 1977). Arntzen (1978) suggested from these findings that good PSII activity is present in the absence of LHC a/b protein complex and the large freeze fracture particles and that the LHC a/b complex is formed by the addition of discrete complexes into the developing membranes.

The chlorophyll b deficient barley and soybean mutants which display no or reduced LHC a/b protein complex and a reduced size of large freeze fracture particles and limited grana formation, further support the association of the LHC a/b complex with the large fracture particles and grana formation (Arntzen, 1978; Boardman et al, 1978).

Leech and coworkers analyzed serial sections of young maize leaves in which the plastids at the leaf base were at the proplastid stage while those in the tip section were fully developed chloroplasts. This was suggested to be a more natural system for development studies since etioplasts do not form under normal light regimes (Leech, 1976). Lipid analysis revealed all the chloroplast acyl lipids were present at all stages. The major changes were associated with sections undergoing rapid membrane formation and grana stacking. The largest increase was again associated with the galactolipids with MG and DG showing respectively a 4 and 2.5 fold increase in tissue sections (Leech et al., 1973) and a 6 and 4 fold increase in isolated plastid fractions (Leese and Leech, 1976). Trans-3-hexadecanoic acid was detected only in PG of the most mature tissue. The galactolipid increase and also the increase in the level of their polyunsaturated fatty acids were much greater than detected with the greening etioplast system. Mackender (1978) using a similar approach has since shown a 2 and 0.2 fold increase respectively in MG and DG and an increase in unsaturation during etioplast development. Photochemical studies revealed a pattern of development similar to the greening etioplast system with the sequential development of PSI and PSII activities (Baker and Leech, 1977).

The development of photochemical activity during chloroplast differentiation has also been studied with several mutants of barley which were capable of chlorophyll synthesis but were impaired in their lamellae differentiation (Smillie *et al.*, 1975). These mutations were of interest in that they corroborated studies on greening leaves showing the sequential development of PSI and PSII photoreductive activities, and also that photoreductive activities can precede the development of the capacity for the photooxidation of water and coupled photophosphorylation.

B. Biosynthesis of Chloroplast Constituents

Plastid Genome

It is now well established that chloroplasts contain their own DNA, RNA, ribosomes and factors required for protein synthesis (Kirk, 1971a). The chloroplast genome is estimated to have the capacity to code for approximately 125 polypeptides of MW 50,000 (Ellis, 1977). Kirk (1967) suggests cytological evidence indicates plastids are always present at all stages during the maturation of the egg cell, providing continuity of the plastids at sexual reproduction. The majority of higher plants display a maternal inheritance pattern of the plastid (Sager, 1972) although biparental transmission is not uncommon. Of 48 genera of angiosperms studied Tillney-Bassett (1975) showed approximately 1/3 displayed at least a trace of biparental transmission. The markers were abnormal plastid colors which were characterized by a non Mendelian inheritance pattern and rapid sorting out to give a variegated appearance. However the mutations have not been localized to the plastid and suitable genetic markers are not available for recombination studies (Sager, 1972; Birky, 1976). Some recombination studies have been carried out with the green alga *Chlamydomonas reinhardi* in which a variety of antibiotic and nutrient markers suggested to be localized to the plastid genome, have been isolated and the experimental techniques developed (Sager, 1972; Gillham, 1974).

Bedbrook and Bogorad (1976) reported a physical mapping technique using restriction endonucleases to fragment chloroplast DNA from *Zea mays*. They were able to ascribe a discrete order to the fragmentation products and proposed a fragment map on which they located the genes for ribosomal RNAs. More recently Coen *et al*. (1977), of the same laboratory, localized the chloroplast DNA segment which directs the synthesis of the large subunit of ribulose bisphosphate (RUBP) carboxylase. The DNA fragments, initially sized on agarose gels were amplified by cloning (incorporation into a bacteria plasmid). One of the cloned fragments directed the synthesis of the large subunit using a rabbit reticulocyte *in vitro* transcription-translation system. Other techniques using the restriction enzymes have recently been used. "Messenger RNA" isolated from etioplasts and greening plastids of corn when hybridized to the fragment DNA's showed a selective increase of some "messenger RNA's", one of which was located on the fragment encoding the LS of RUBP carboxylase and another suggested to be the fragment encoding a 34,500 dalton chloroplast membrane protein (Bedbrook *et al.*, 1978; Grebanier *et al.*, 1979). Bottomley and Witfeld (1979) used an indirect approach, spinach chloroplast DNA was fragmented independently with several restriction endonucleases and the fragments transcribed/translated using the cell free *E. coli* system. The polypeptide maps produced from the restriction fragments were compared with those produced with whole chloroplast DNA. Bands missing on any of the fragment DNAs were suggested to have their cistrons inactivated by the cleavage site of the restriction enzyme.

The chloroplast genome is considered polyploid containing several copies per chloroplast (Kung, 1977). The polyploid nature and the maternal inheritance pattern are known to be conservative forces for genetic variability (Grun, 1976). The chloroplast DNA of several higher plants studied is known to have a very constant base composition and density even though the composition and density of nuclear DNA varies markedly among different species (Kirk, 1971a). However fragmentation patterns produced by the restriction enzyme technique showed the number of bands in common varied considerably from genus to genus. The similarities seemed to correlate with how closely the species were related as determined by their ability to form viable hybrids (reviewed by Kung, 1977). On this matter Bogorad (1975) had earlier theorized that although the organelles appear to have a common function, the genes which specify their composition could be distributed differently among the genomes of the nucleus, mitochondria and plastids in different taxonomic groups.

Protein Synthesis

This section will briefly include some experimental approaches which have suggested the joint participation of both the plastid and nuclear genomes in chloroplast formation. Polypeptide patterns produced by coupling chloroplast DNA with an *in vitro* transcription/ translation system suggest, from preliminary results, that close to 30% of the total chloroplast genome can be expressed in terms of protein product (Bottomley and Whitfeld, 1979). This approach as described earlier provided the information that the LS of RUBP carboxylase and a suggested 34,500 dalton membrane polypeptide are encoded on the chloroplast genome. Using a similar approach mRNA, assumed to be of nuclear origin (Poly-A containing RNA) isolated from greening barley or duckweed coupled with the in vitro wheat germ translation system synthesize several polypeptides of which precursors to the apoprotein of the LHC a/b protein and the small subunit of RUBP were identified (Apel and Kloppstech, 1978; Tobin, 1978)

Protein synthesis by isolated chloroplasts and in vivo studies using inhibitors specific for cytoplasmic and chloroplast ribosomes have also shown that the LS of RUBP carboxylase was synthesized in the chloroplast and the small subunit in the cytoplasm (Ellis, 1977). Using a similar system three of the five subunits of the chloroplast coupling factor and some of the polypeptides associated with CPI are suggested to be translated on the chloroplast ribosome while some polypeptides associated with the LHC a/b protein complex were synthesized in the cytoplasm (Ellis, 1977). The selectivity of the inhibitors is due to the prokaryotic nature of the chloroplast ribosome and cautions and guidelines for their use and interpretation have been outlined (McMahon, 1975; Grun, 1976; Ellis, 1977) .

Studies with interspecific genetic markers for chloroplast ribosomal proteins and the large and small subunits of RUBP carboxylase have also indicated an intergenomic involvement in chloroplast protein synthesis (Bogorad, 1975; Kung, 1977; Gillham, 1978). The markers were altered peptide migration patterns between species which showed either a biparental or maternal inheritance pattern in reciprocal crosses.

Chlorophyll Biosynthesis

The complete biosynthetic pathway from 5-ALA to chlorophyll is suggested to occur within the plastid (Bogorad, 1976). However efforts to demonstrate 5-ALA formation from glycine and succinyl CoA in plants as in the porphyrin synthetic pathway of animals and bacteria have not been successful. Studies on the precursors for 5-ALA formation were greatly facilitated by the use of levulinic acid as a competitive inhibitor of ALA-dehydrase by Beale and coworkers in the early 1970's (Beale, 1978). The inhibitor which can be employed in vivo causes a partial block in tetrapyrrole synthesis resulting in the intracellular accumulation of 5-ALA. This accumulation is stoichiometrically related to the decrease in chlorophyll formation and thus permits a more direct study for initial precursors for chlorophyll formation. Five-carbon compounds glutamate, glutamine and α-ketoglutarate were found to be relatively good precursors and were incorporated intact into ALA suggesting an alternate pathway to the previously sought ALA synthetase (Beale, 1978). Recently, isolated plastids from immature spinach leaves (Gough and Kannangara, 1976), greening barley and maize (Kannangara and Gough, 1977) and greening cucumber cotyledons (Weinstein and Castelfranco, 1978) were found capable of 5-ALA formation from the 5-C precursors. This activity was associated with the intact plastids and not with mitochondria and microsome cell fractions (Gough and Kannangara, 1976). The activity was further localized to stroma preparations following disruption of isolated plastids (Gough and Kannangara, 1977) and co-purified with a polypeptide with an approximate MW of 67000 daltons (Kannangara and Gough, 1979). The intermediate and enzymes associated with 5-ALA from the 5-C precursors however have yet to be demonstrated (Beale, 1978).

The biosynthetic steps subsequent to 5-ALA formation are better characterized (reviewed by Bogarad, 1976). The associated enzymes up to protoporphyrin synthesis are suggested to be located in the stroma fraction while those subsequent are membrane associated (Smith and Rebeiz, 1979). Exogenous 5-ALA is readily incorporated into protoporphyrin by the stroma fraction from greening cucumber cotyledons and the membrane fraction converts exogenous protoporphyrin, solubilized with methanol, into Mg-protoporphyrin (Smith and Rebeiz, 1979). Griffiths (1974) had earlier shown that membrane fractions of barley etioplasts were capable of converting exogenous Pchlide, solubilized in a mixture of methanol and sodium cholate by brief sonication, to chlorophyllide. The intraplastid location of the conversion of Mg proto-porphyrin to Pchlide has not been demonstrated (Smith and Rebeiz, 1979).

As previously described, an active Pchlide-holochrome complex has been isolated from dark grown bean and barley leaves. The bean complex was soluble in aqueous buffers whereas the barley complex was solubilized with the detergent mixture, saponin (Henningsen and Kahn, 1971;

Stummann, 1978). The aqueous solubility of the bean holochrome complex is now suggested to be due to the high lipase activity in bean homogenates (Boardman _et al._, 1978). Thus the complex also appears to be membrane associated.

Evidence presently supports two theories of phytylation (Bogorad, 1976; Liljenberg, 1977). The enzyme chlorophyllase, which requires detergents or acetone for solubilization and thus is presumably membrane bound, catalyses the hydrolysis of chlorophyll a and b to the respective chlorophyllides and free phytol. Some indirect evidence suggests chlorophyllase acts synthetically, however other findings indicate phytylation probably occurs by a phytol pyrophosphate intermediate by analogy with the terpenoid biosynthesis (Bogorad, 1976; Liljenberg, 1977).

Preliminary studies have shown 5-ALA formation is not inhibited by chloramphenicol, an inhibitor of chloroplast ribosome activity, but is inhibited by cycloheximide, a potent inhibitor of protein synthesis on cytoplasmic ribosomes suggesting the enzymes are not synthesized within the plastids (Gough, 1978; Kannangara and Gough, 1979). The identification of genes controlling chlorophyll synthesis from 5-ALA to Pchlide was facilitated by the discovery of Granick (1967) that externally applied 5-ALA is readily taken up in dark grown leaves and converted into Pchlide (reviewed by Wettstein _et al._, 1971). 5-ALA synthesis is subject to feed-back inhibition by Pchlide or Pchlide precursors present in low amounts, thus feeding with 5-ALA results in the accumulation of high levels of Pchlide or precursor intermediates if a blockage occurs in dark grown seedlings. By feeding chlorophyll deficient mutants 5-ALA Wettstein *et al.* (1971) demonstrated mutations controlling the steps between protophorphyrin IX and Pchlide were inherited in a Mendelian pattern in several barley mutants. However it is not known if the genes code for enzymes catalysing the steps in chlorophyll synthesis or the membrane constituents which bind the enzymes or their substrates (Wettstein *et al.*, 1971). More recently regulatory genes which relax the repression of 5-ALA synthesis in dark grown seedlings has been described which also show a Mendelian inheritance pattern (Wettstein *et al.*, 1974; Kahn *et al.*, 1976).

Carotenoid Biosynthesis

All green tissue of higher plants of diverse taxonomic types and differing habitats contain the same major carotenoids, β-carotene, lutein, violaxanthin and neoxanthin, which have been suggested to be located exclusively in the chloroplast (Goodwin, 1976). Goodwin (1976) suggests this ubiquitous pattern may indicate any mutations which significantly alter the carotenoid composition would be lethal.

Carotenoids are one group of several prenyllipids found in higher plants which are biosynthesized by the terpenoid pathway, that is built up from C-5 isoprene units (Britton, 1976; Davies, 1977; Goodwin, 1977). The other higher plant prenyllipids include plant sterols and mixed terpenoids. The mixed terpenoids include the isoprenoid side chains of the benzo- and naphthoquinone derivatives, and the phytol portion of the chlorophyll molecule (Lichtenthaler, 1977; Goodwin, 1977). All the prenyllipids except sterols, ubiquinone and tocopherols are located specifically in the plastids. Ubiquinone is found specifically in the mitochondria and the sterols are present in all membranes (Goodwin, 1977). Within the plastid the prenyl constituents are located mainly on the lamellar membrane whereas the prenyl quinones function as potential electron carriers (Lichtenthaler, 1977), and the carotenoids and chlorophyll are associated with the photosystem reaction centers and light harvesting complexes (Thornber, 1975). β-Carotene has been isolated in a protein complex, however it is not known if this represents its in vivo association (Ke, 1971). The carotenoids are also associated with the plastid envelope (Douce et al., 1973; Jeffery et al., 1974) and the quinones with the plastoglobuli (Goodwin, 1977).

The initial steps in the biosynthesis of the prenyllipids are believed to be the same, the formation of isopentyl pyrophosphate from mevalonic acid and the successive additions of isopentyl pyrophosphate to form a C_{15} intermediate. From here the pathways diverge to form the many prenyllipids (Goodwin, 1977). Only the carotenoid pathway will be briefly described. Labelling studies in combination with inhibitors and mutations have been used over the past 20 years to identify the intermediates (Britton, 1976; Davies, 1977). Briefly C_5 units are added sequentially to form C_{20} (geranyl geranyl pyrophosphate). Two C_{20} units are joined to form C_{40} phytoene. Most naturally occurring carotenoids of higher plants are C_{40} tetraterpenes (Britton, 1976). Phytoene is sequentially desaturated to the colored carotene lycopene which is converted into cyclized carotenes, (α-carotene and β-carotene). The xanthophylls, lutein, violaxanthin and neoxanthin are suggested to be formed by the addition of hydroxyl and epoxy groups to the cyclic carotenes (Britton, 1976; Davies, 1977). Limited studies have been carried out on the enzymology of the system. A major technical problem has been the insolubility and instability of long chain hydrocarbon intermediates in enzyme preparations such that most labelling studies have required the use of early precursors (Davies, 1977).

Preliminary evidence suggests the plastid has the ability to synthesize the carotenoids and the terpenoid side chain of chlorophyll and the plastid quinones, whereas the sterols and ubiquinones are suggested to be synthesized elsewhere in the cytoplasm (Bickel and Schultz, 1976; Goodwin, 1976; Lichtenthaler, 1977). Nuclear mutations of both maize and tomato indicate nuclear genome control of the desaturation and cyclization systems (Kirk, 1967; Bachmann *et al.*, 1973; Davies, 1977; Goodwin, 1977).

Acyl Lipid Biosynthesis

Findings to date indicate, contrary to pigment biosynthesis, that the chloroplast is not capable of complete acyl lipid biosynthesis, but requires enzyme systems associated with both the plastid and cytoplasm (Givan and Harwood, 1976; Leech and Murphy, 1976; Stumpf, 1977). The frequently used system to study the pathways and kinetics of lipid synthesis has been the incorporation of ^{14}C-acetate or $^{14}CO_2$ using either intact leaf tissue or subcellular fractions. Intermediate steps, such as fatty acid elongation, desaturation and complex lipid formation have been further characterized using more complex substrates.

Studies with greening etiolated tissue (Appelqvist *et al*., 1968; Kannangara *et al*., 1971; Panter and Boardman, 1973) and developing leaf segments (Hawke *et al*., 1974a; Hawke *et al*., 1974b; Slack and Roughan, 1975; Williams *et al*., 1975; Bolton and Harwood, 1978) showed the incorporation of acetate or CO_2 was most active during the period of chloroplast development. $^{14}CO_2$ was rapidly incorporated into the galactose portion of galactolipids accounting for over 90% of the label (Williams *et al*., 1976) however the labelling patterns into the fatty acids of the chloroplast acyl lipids were similar to those of ^{14}C-acetate (Slack and Roughan, 1975; Heinz and Harwood, 1977).

Analysis of subcellular fractions of green tissue suggested that the chloroplast is the major site of fatty acid synthesis (Smirnov, 1960; Mudd and McManus, 1962; Stumpf and James, 1963). More recently using cell fractions prepared from spinach leaf protoplasts, which resulted in little organelle disruption and a sensitive radioimmunoassay method to quantitate ACP, it was concluded that the chloroplast is the sole site of cellular _de novo_ fatty acid biosynthesis (Ohlrogge _et al_., 1979). The plastids in non-photosynthetic tissue: avocado mesocarp (Weaire and Kekwick, 1975) and developing castor bean endosperm (Zilkey and Canvin, 1969; Nakamura and Yamada, 1974; Vick and Beevers, 1978) are also suggested to be the major site of _de novo_ cellular fatty acid synthesis. However, Harwood (1975) found that the fat fraction of developing castor bean endosperm and Mazliak (1977) that mitochondria isolated from non--photosynthetic tissue (potato tuber, cauliflower florets, bean and lupin roots) and Bolton and Harwood (1977) that microsomes from germinating pea seedlings were also active in fatty acid synthesis.

In both _in vivo_ and _in vitro_ studies on green leaves the incorporation patterns are not representative of the endogenous fatty acid levels. The intact tissue incorporated label into polyunsaturated fatty acids although at much lower levels than expected from the endogenous content, while the isolated plastid fractions incorporated label almost entirely into palmitic and oleic acid. The lower incorporation rates with intact tissue are perhaps partially explained by the low incorporation into the galactolipids which are rich in the polyunsaturated fatty acids (Hawke _et al._, 1974a). Pulse-chase experiments with ^{14}C-acetate suggest a time lag in the labelling of the acyl moieties of MG and DG. Label occurs rapidly in PC-18:1, which declines during the chase period and accumulates in 18:2 and 18:3 in MG and to a lesser extent in DG (Slack and Roughan, 1975; Williams _et al._, 1976; Slack _et al._, 1977; Wharfe and Harwood, 1978). In fact the labelling of the polyunsaturated fatty acids of the galactolipids was still increasing after 72 h of chase (Williams _et al._, 1976). Also studies with greening cucumber cotyledons suggest the C-18 desaturase activities may be selectively induced during the development of the chloroplast membranes (Murphy and Stumpf, 1979). Activities from the more mature tissue were at a much lower level and are suggested to keep pace with membrane turnover (Murphy and Stumpf, 1979). Thus both the state of maturity of the tissue and the often used short incorporation periods could partially account for the low labelling of polyunsaturated fatty acids in intact tissue.

The distribution of the labelled acyl groups among the lipid classes also differed markedly between the intact tissue and the isolated plastid. Using intact tissue the polar lipids accumulated 85-90% of the acyl label from ^{14}C-acetate (Roughan _et al._, 1976) whereas with isolated plastids free fatty acids contained 70-80% of the activity of which oleic acid (18:1) constituted 76-90% of the free fatty acids synthesized (Hawke _et al._, 1974b; Roughan _et_ al., 1976). More recently it was shown that incubation of intact plastids with CoA and high ATP concentrations shifted the label from predominantly free fatty acids to diacylglycerols and acyl CoA's (Kleinig and Liedvogel, 1979). The inability of isolated chloroplasts to synthesize polyunsaturated fatty acids appears to be due to their interdependence on other cellular components for further lipid synthesis. If the maize chloroplast fraction was substituted with the non chloroplast particulate fraction an increased biosynthesis of polyunsaturated fatty acids resulted (Hawke et al., 1974b). Also cell fractionation immediately following ^{14}C feeding showed the rapidly labelled PC-18:1 was associated with a non-chloroplast membrane fraction sedimenting at 10,000g and 140,000g (Slack and Roughan, 1975; Simpson and Williams, 1979). During the chase period the label declined in the 'microsomal' membrane fraction and increased in the MG-acyl lipid in the chloroplast fraction (Slack and Roughan, 1975).

Stumpf (1975; 1977) and coworkers have published an extensive series of papers on the biosynthesis of fatty acids in higher plants. The primary product of de novo synthesis in the chloroplast is palmitoyl-ACP which may be further elongated to stearoyl-ACP. Also stearoyl-ACP desaturase and oleoyl-ACP hydrolase occur within the chloroplast, which function jointly to form oleic acid, the principal product of fatty acid synthesis in the isolated chloroplast (Ohlrogge et al., 1978). The de novo synthesizing enzymes as well as the elongation and steroyl acyl desaturase enzymes are suggested to occur in the stroma phase (Stumpf, 1977). The enzymes of the *de novo* synthesis complex have not been isolated although they are considered to be present as discrete separable proteins since they are not sedimented by high gravitational force (Stumpf, 1977). This is similar to the prokaryotic system and differs from the multienzyme complex of animals and yeast whose components are not separable (Hitchock and Nichols, 1971).

Long chain acyl CoA synthetase activities for 16:0 and 18:1 were shown to be associated with the chloroplast envelope fraction (Joyard and Douce, 1977; Roughan and Slack, 1977). The acyl-CoA's serve as the substrates for further lipid synthesis: the desaturation and acylation of polar lipids (Stumpf, 1977). Further fatty acid desaturation is somewhat less understood. The microsomal preparations of both leaf tissue (Slack *et al*., 1976; Dubacq *et al*., 1976) and developing safflower seeds (Vijay and Stumpf, 1971) possess oleoyl desaturase activity forming 18:2. The nature of the substrate for the desaturase enzyme is not agreed upon, however the 18:2 was rapidly incorporated into PC. The chloroplast fraction was shown to have the potential to form 18:3 by the sequential desaturation of 18:2 (Tremolieres and Mazliak, 1974) or the elongation of 16:3 in 16:3 plants, such as spinach (Jacobson *et al*., 1973; Vance and Stumpf, 1978). Thus in the synthesis of polyunsaturated fatty acids the missing link is the synthesis of 18:3 from 18:2. Slack et al. (1977) using ^{3}H glycerol and ^{14}C acyl groups have shown the complete diacylglycerol portion of PC is transferred into MG. Slack and Roughan suggest their results indicate acyl transfer does occur between the various cellular compartments with PC serving as the major carrier. However it is not clear which acyl groups are transferred from PC to the galactolipids since the label lost from PC is accumulated in MG in a more unsaturated form. Preference of the galactosylating enzyme, forming MG from UDP-galactose and diglycerides, for highly unsaturated diglycerides has however, been reported (Mudd et al., 1969; Williams et al., 1976).

Enzyme systems that support a lipid transfer have been described. The chloroplast envelope has the capability to form phosphatidic acid from long chain acyl CoA's and glycerol-3-P (Joyard and Douce, 1977). PA was shown to play a central role in the phospholipid biosynthetic pathway (Kates and Marshall, 1975). The exchange of phospholipids between plant cellular organelles was shown to be stimulated by a soluble protein termed phospholipid exchange protein (PLEP) (Kader, 1975; Mazliak, 1977; Douady et al., 1978) and by 'vesiculation' from the endoplasmic reticulum in the formation of glyoxysomes in germinating castor bean endosperm (Lord, 1976; 1978).

Several workers have reported that galactolipid synthesis is concentrated in the chloroplast envelope fraction (Douce, 1974; van Hummel et al., 1975; Joyard and

Douce, 1977; van Besouw and Wintermans, 1978; 1979; Williams et al., 1979). Two different enzymes for the galactosylation of MG and DG have been suggested (Ongun and Mudd, 1968; Besouw and Wintermann, 1978). The difference in the nature of acyl groups of the diglyceride moiety has not been explained. Suggestions to account for the differences have included substrate specificity, separate pools of MG, acyl exchange and desaturation after incorporation of acyl groups into the lipid (Mudd and Garcia, 1975; Heinz, 1977).

The biosynthesis of the phospholipids: PI (Kates and Marshall, 1975), PC (Devor and Mudd, 1973), PE (Macher et al., 1974; Marshall and Kates, 1974) and the major chloroplast phospholipid PG (Kates and Marshall, 1975) have been localized primarily to the microsomal fraction of spinach leaves. The details and the intracellular location of SL biosynthesis have not been described (Harwood, 1975a; Harwood and Nicholls, 1979).

The localization of biochemical activity to intracellular components is subject to considerable variation. The difficulties in the preparation of subcellular fragments and the use of biochemical and morphological markers to establish the degree of purity have been described in several recent reviews (Heinz, 1977; Leech and Murphy, 1976; Stumpf, 1976; Quail, 1979). For example it is technically difficult to prepare a cytosol fraction from plant leaf cells which is not contaminated by the stroma fraction of disrupted plastids. Also soluble enzymes released may be adsorbed on membrane surfaces or incorporated into vesicles formed by the disruption of endoplasmic reticulum or chloroplast envelopes (Stumpf, 1976). The chloroplast envelope membrane fragments probably sediment with other organelles and membrane fragments between 4000g and 144000g (Leech and Murphy, 1976; Williams _et al._, 1979). Also balance sheets of enzyme activities among subcellular fractions could easily be altered by the unequal distribution of lipid degrading activities in these fractions (Heinz, 1977; Douady _et al._, 1978).

Few studies are available on the genetics of lipid synthesis. Breeding programs for modified fatty acid composition in seed oils suggest nuclear control of elongase and desaturase enzymes (Downey and McGregor, 1976). Studies on wheat kernels indicate that the gene or genes localized to a segment of chromosome 5 control the relative levels of MG and DG in wheat endosperm (Hernandez-Lucus _et al._, 1977). A nuclear mutant has been described which releases the controls on MG synthesis in greening barley seedlings. Even though the mutant contains only 25% of the chlorophyll content present in the normal its incorporation of acetate into MG surpasses the normal by 50% (von Wettstein _et al._, 1971). The light induced increase in desaturase activity in greening cucumber cotyledons was inhibited by cycloheximide suggesting the increase in the desaturase enzymes is dependent upon protein synthesis by the cytoplasmic ribosomes (Murphy and Stumpf, 1979).

F. Acetyl Coenzyme A Carboxylase

Multienzyme Complexes from Animal and Yeast Tissue

Acetyl CoA carboxylase was initially identified in the late 1950's using avian liver extracts as one of two essential enzyme complexes required for long chain fatty acid synthesis. The other complex was later shown to be the fatty acid synthetase multienzyme complex. The synthesis of fatty acids from acetyl CoA was shown to be bicarbonate dependent. This led subsequently to the finding that malonyl CoA was the product of the ATP-dependent carboxylation of acetyl CoA and that the fatty acid synthetase complex utilized malonyl-CoA for reductive elongation of acyl-CoA's (reviewed by Lane *et al*., 1974). Acetyl CoA carboxylase has now been purified from a variety of animal tissues, yeast and microorganisms. All the carboxylases studied were shown to contain defineable subunits, however the animal and yeast complexes required SDS and urea treatment for dissociation which resulted in the inactivation of the subunits. The *E. coli* enzyme complex was however readily dissociable into active subunits and the partial reactions have been primarily defined by the study of the *E. coli* systems (Volpe and Vagelos, 1976).

Escherichia coli Enzyme Complex

The *E. coli* complex was shown to contain three proteins, biotin carboxylase (BC) biotin-carboxyl carrier protein (BCCP) and carboxyltransferase (CT) which were readily separated by conventional protein fractionation techniques. The proteins were found to catalyse the two half reactions as shown by Volpe and Vagelos (1976).

$$ATP + HCO_3 + BCCP \xrightarrow{BC/Mg,Mn} CO_2\text{-}BCCP + ADP + Pi$$

$$CO_2\text{-}BCCP + acetyl\text{-}CoA \xrightarrow{CT} BCCP + malonyl\text{-}CoA$$

The carboxylase was previously found to be a biotin enzyme since avidin blocked its activity (Lane et al., 1974). The mechanism of action of acetyl CoA carboxylase has been shown to be similar to that of other biotin enzymes (Vagelos, 1974; Visser and Kellogg, 1978; Wood and Borden, 1977). The biotin serves as the covalently bound prosthetic group, binding to just one of the polypeptides which is termed the biotin carboxyl carrier protein (Vagelos, 1976). The BCCP plays a central role in the carboxylation of acetyl CoA and as shown above it is carboxylated to form CO_2-BCCP in the biotin carboxylase reaction and the carboxyl group is transferred to acetyl-CoA to form malonyl CoA by the carboxyltransferase catalyzed reaction.

Wheat and Barley Embryo Enzyme Complexes

Malonyl CoA was also shown to be a highly reactive substrate for fatty acid synthesis in plant tissue and acetyl CoA carboxylase was considered to be the key enzyme involved in its synthesis (Stumpf, 1976). Acetyl CoA carboxylase from wheat germ (Hatch and Stumpf, 1961; Heinstein and Stumpf, 1969) and barley embryo extracts

(Brock and Kannangara, 1976) was shown to be a completely soluble protein and has been purified 1000-fold to a specific activity of 6.3 and 7.4 umoles $H^{14}CO_3$ incorporated per mg protein per min, respectively. The highly purified wheat germ enzyme was partially dissociated into two protein constituents on sucrose density gradients followed by ultracentrifugation or molecular sieving on Sepharose 4B. The partial activities of the two proteins suggested the BC and BCCP separated as one complex and CT as the other (Heinstein and Stumpf, 1969). The MW of the barley enzyme complex was estimated by gel filtration chromatography as 610,000 daltons. The barley complex separated into 3 bands on polyacrylamide gels of which only one contained biotin and corresponded to a MW of 21,000 daltons. The biotin polypeptide was identified as the BCCP since it was able to function as the carboxyl carrier with purified _E. coli_ BC and CT (Brock and Kannangara, 1976). The _E. coli_ BCCP was found to have a MW of 22,500 Daltons. Earlier reports of 9,100 Daltons were shown to be due to the action of proteases (Fall and Vagelos, 1972).

Chloroplast Enzyme Complex

The location of the enzyme within the chloroplast has been determined by indirect methods. Intact chloroplasts readily incorporate acetate into palmitic and oleic acid in a CO_2 requiring reaction suggesting the acetyl CoA carboxylase is functioning effectively. However, upon disruption of the chloroplast, acetate and acetyl CoA served as very poor substrates but malonyl CoA was readily incorporated into fatty acids (Brooks and Stumpf, 1966). These findings suggested that the acetyl CoA carboxylase was absent or inactive in such preparations. The addition of phosphorylated compounds which serve as substrates for the reductive carbon cycle indicated the disrupted chloroplast fraction was capable of performing carboxylations associated with the cycle suggesting a selective inhibition for acetyl CoA carboxylase (Burton and Stumpf, 1966). Decarboxylation of malonyl CoA and deacylation of acetyl CoA were ruled out. The presence of an inhibitor was tested for by the addition of the disrupted chloroplast fraction to a preparation of wheat germ carboxylase (Burton and Stumpf, 1966). The chloroplast preparation markedly depressed the wheat germ activity and the inhibition was shown to be independent of the reaction time and heat stable (to boiling water for 5 min) and also not removed with papain digest suggesting it was non enzymatic (Burton and Stumpf, 1966). The inhibitory effect was lost upon ashing indicating heavy metals were not the cause (Burton and Stumpf, 1966). The above study was conducted on lettuce chloroplasts. An inhibitory effect was also suggested from studies with barley plastids. Mild activity was detected on plastids isolated from etiolated seedlings during initial illumination but the activity declined quickly during the first 12 h of greening (Kannangara _et al_., 1971).

Kannangara and Stumpf (1972) postulated that the chloroplast enzyme properties were similar to those of *E. coli*. Incubation of disrupted spinach chloroplasts with ATP, ^{14}C-bicarbonate and $MgCl_2$ and $MnCl_2$ followed by filtration through Sephadex G-50 revealed the formation of protein bound-$^{14}CO_2$ suggesting the constituents BC and BCCP which are necessary for the formation of BCCP-CO_2 were functional. The presence of functional BC and BCCP within the plastids was also indicated by the study in which the chloroplast assay system was supplemented with the *E. coli* carboxyltransferase and good malonyl CoA formation occurred. However very high concentrations of *E. coli* CT were required for maximum activity, 1.5 mg per 31 ug chlorophyll equivalent enzyme preparation (Kannangara and Stumpf, 1972). The location of the enzyme subunits within the chloroplast was also studied in this work. The separated stroma and lamellar fractions were themselves ineffective in binding $^{14}CO_2$. Both fractions were required for BCCP-$^{14}CO_2$ formation. Subsequent separation of the fractions showed the lamellar fraction contained the BCCP-$^{14}CO_2$ and the label was readily incorporated into malonyl CoA upon the addition of acetyl CoA and *E. coli* CT. Thus the BC constituent was concluded to be a stromal enzyme (Kannangara and Stumpf, 1972). Addition of 0.06 M to 0.1 M sodium bicarbonate to the disruption buffer enabled the detection of very low incorporation of 1-^{14}C acetyl CoA into malonyl CoA (Kannangara and Stumpf, 1972), by perhaps stabilizing the CT constituent (Stumpf, 1975; 1977). This indicated the complete acetyl CoA carboxylase enzyme system occurs within the plastid. The CT constituent was further localized to the stromal fraction by testing the stromal and lamellar fractions for activity using 1-^{14}C acetyl CoA and E. coli BC and BCCP (Ea) (Kannangara and Stumpf, 1972). Thus this study concluded that the BCCP was membrane bound while BC and CT were soluble in the stroma.

A study of the biotin distribution within chloroplasts of a number of higher plant species showed considerable variability in the per cent soluble and membrane bound biotin. Two extremes were tobacco and barley which contained 56% and 80% respectively of their biotin content in the membrane bound form (Kannangara and Stumpf, 1973). Further study of non membrane bound biotin revealed that most of it was not precipitated by heat denaturation followed by centrifugation at 110,000g and was taken as non protein associated biotin (Kannangara and Stumpf, 1973). Biotin protein was primarily in the soluble form in barley embryos and was shown to become predominantly membrane bound in 4 day seedlings (Kannangara and Stumpf,. 1973). More recently Kannangara and Jensen (1975) were able to incorporate ^{14}C-biotin into a chloroplast lamellar protein by aseptically culturing barley embryos in a medium containing the labeled biotin. Little biotin was shown to be metabolized in this study. Disc gel electrophoresis of the lamellar fraction showed a single radioactive spot with an apparent MW of 21,000 daltons. The polypeptide retained its functional form and was shown to function as a carboxyl carrier in the E. coli acetyl CoA carboxylase assay, E. coli BC + CT + lamellar BCCP, to form malonyl CoA (Kannangara and Jensen, 1975).

Other Chloroplast Enzyme Complexes

Recently two enzyme complexes have been implicated in the generation of the substrates for the acetyl CoA carboxylase complex . Using chloroplast suspensions isolated from young expanding spinach leaves and displaying 70 to 90% intactness, as judged by phase contrast microscopy and ferricyanide reduction and capable of high rates of photosynthetic CO_2 fixation, Murphy and Leech (1977) first demonstrated isolated plastids could incorporate photosynthetically fixed CO_2 into acyl lipids. The pattern of labelling was similar to that described using acetate. It was thus inferred chloroplasts were capable of acetyl CoA synthesis. Earlier studies had suggested a shuttle mechanism involving extrachloroplast enzymes to supply the chloroplast with acetyl CoA (Sherratt and Givan, 1973). A possible pathway for biosynthesis of acetyl CoA in the chloroplast was postulated by comparison of the labelling patterns of different postulated precursors and isotope competition studies (Murphy and Leech, 1978). Their findings were consistent with the proposed pathway:

HCO_3-->3PGA--->PEP--->pyruvate--->acetylCoA--->fatty acids

This is also in agreement with the proposal of Yamada and Nakamura (1975) who showed PGA and pyruvate were effective substrates using a ^3H_2O incorporation system whereas malate, citrate, OAA, glyoxylate and glycollate were inadequate. Their results further indicated that the principal path for pyruvate was the decarboxylation of pyruvate to acetyl CoA consistent with the activity of the pyruvate dehydrogenase complex. Pyruvate dehydrogenase complex activity has been found associated with the proplastid fraction of developing castor bean endosperm (Reid *et al.*, 1977) and the acetyl CoA generated is probably channeled into long chain fatty acids (Simcox *et al.*, 1977).

Wolpert and Ernst-Fonberg (1975) isolated a multienzyme complex from *Euglena gracilis* by biotin affinity chromatography. The enzyme complex contained three enzyme activities, phosphoenolpyruvate carboxylase, malate dehydrogenase and acetyl CoA carboxylase. They proposed that the complex enabled a relatively high concentration of HCO_3 to be created and in this way the CO_2 captured by the PEP carboxylase is channeled specifically to the acetyl CoA carboxylase.

III. LIPID CHANGES DURING GREENING OF BARLEY SEEDLINGS

MATERIALS AND METHODS

A. Plant Material

Barley (*Hordeum vulgare* cv. Gateway) and its chlorophyll deficient mutant were used in this study. The mutation is thought to be a spontaneous one since it arose in the water control of a chemical mutagenic study (Miller, 1965). It is described as being of the virescens type since seedlings which were originally pale yellow-green on emergence developed near normal pigment levels with age (Maclachlan and Zalik, 1963). Walker et al. (1963) concluded the mutation involved a single recessive nuclear gene and this was confirmed with reciprocal crosses (Stephansen and Zalik, 1971).

Seeds of Gateway and the mutant were surface sterilized with 5% sodium hypochlorite for 15 min, then rinsed several times with distilled water and grown in vermiculite. The two lines were grown simultaneously under continuous light at 600 ft-c and 20 C. Seedlings were harvested at intervals from 4 to 8 days after planting. The apical 3 cm leaf segments were used for lipid and pigment analysis except, when indicated, an additional 3 cm segment towards the basal region and adjacent to the apical segment was also studied.

B. Solvents

All solvents were of analytical reagent grade. Acetone, chloroform, hexanes, ligroine (petroleum ether bp 63-75 C), methanol, and n-propanol were redistilled before used. Phenol used for sugar determinations was redistilled and allowed to crystallize.

C. Lipid Extraction

Seedlings were harvested at 4, 6, and 8 days after planting and weighed in capped metal tins. Segments from 50 leaves, approximately 0.8 g, were required for the 4 day analysis, however 30 leaves, approximately 0.5 g, provided adequate sample for the 6 and 8 day analyses. The leaf segments were wrapped in cheese cloth and steamed for 10 min to denature lipase enzymes (Yang et al., 1967; Roughan and Boardman, 1972). The samples were then extracted with approximately 15 ml chloroform/methanol (C:M) (2:1, v/v) using a Ten Broeck tissue grinder. The homogenate was transferred to a stainless steel test tube and centrifuged at 10,000g for 10 min. The pellet was resuspended in C:M (2:1,v/v) and the suspension filtered through Whatman #1 filter paper with several washings. The filter paper containing the pigment free residue was saved for nitrogen determinations. The filtrate plus initial supernatant were pooled and made to volume (25 ml). A 2 ml aliquot was withdrawn for chlorophyll estimations and 20 ml for lipid estimations. The non-lipid contaminants were removed using the method of Williams and Merrilees (1970), which utilizes the ability of Sephadex G-25 to swell in water and absorb aqueous soluble contaminants. For the phase separation methods of Folch et al. (1957) and Bligh and Dyer (1959) separations were obtained by low speed centrifugations. Using the Sephadex method, 1 gm of Sephadex G-25 was added to each lipid extract followed by 0.2 ml water to facilitate swelling. Initial samples prepared without the addition of water showed contaminants around the origin of thin layer chromatograms. The solution was concentrated at room temperature until no free liquid remained using a rotary vacuum evaporator with the receiver cooled by liquid nitrogen. The Sephadex was resuspended in chloroform and concentrated again, this ensured complete absorption of the aqueous soluble compounds (Williams and Merrilees, 1970). The sample was again resuspended in chloroform and the suspension poured into a narrow chromatographic column (1 cm ID) with the end drawn to a capillary and the opening layered with glass wool. Lipids were washed from the Sephadex with 100 ml chloroform. Monitoring further washes containing methanol (C:M, 2:1v/v) by thin layer chromatography (TLC) revealed only faint traces of some lipid components but also showed contaminants about the origin. A yellow pigment remained in the column. It was not eluted with methanol but was removed with water. The 100 ml chloroform wash was concentrated on a rotary vacuum evaporator and made to volume (2 ml) with chloroform. For total lipid weight determination 600 ul samples in duplicate were transferred to pre-weighed vials and concentrated to dryness with nitrogen. The samples were further dried over silica gel in a vacuum desiccator for approximately 3 days for constant weight determination.

D. Separation of Polar Lipids

The polar lipids were separated by two-dimensional thin layer chromatography (2D TLC) using the solvent system outlined by Allen and Good (1971). Thin layer glass plates (20 x 20 cm) were coated with a 250 um layer of silica gel HR (Merck). They were allowed to dry at least 3 h at room temperature before activation and were activated just prior to use at 110 C for 1 h and cooled in a desiccating chamber.

A 200 ul aliquot of the total lipid extract, containing 0.7 to 1 mg (gravimetric estimation) was applied as a single spot to the lower left corner 2 cm from either edge. During sample application the TLC plate was maintained under nitrogen atmosphere using a special fabricated applicator box which enabled a continuous stream of nitrogen to be passed over the plate.

The chromatography tanks were filled to a depth of 1.5 cm with the developing solvent and the tanks which were lined with filter paper were tilted to moisten the filter paper liner. The solvents were prepared fresh for each run. In the first direction the chloroform:methanol:water mixture (65:25:4,v/v) was allowed to run approximately 15 cm. The plates were removed and dried in a vacuum oven at room temperature for 15 min. The vacuum was released under nitrogen and the plates were immediately chromatographed in the second direction with the chloroform:methanol:-isopropylamine:ammonia (65:35:0.5:5, v/v) solvent system.

E. Identification of Phospholipids, Glycolipids and Gramine

Spraying the plate lightly with 50% sulfuric acid and charring in an oven at 180 C for 15 min outlined all the lipid spots on the TLC plate. Authentic samples, fatty acid analysis, infra red spectral analysis, specific color tests and comparisons to the migration patterns shown in published chromatograms were used to identify lipid classes and individual lipid spots

F. Lipid Quantification

Quantification of the lipid from the normal and mutant seedlings was based on their fatty acid content using an internal fatty acid standard (Kuksis, 1966; Allen and Good, 1971). The lipids were also quantified on the basis of their sugar (Roughan and Batt, 1968) and phosphorus (Bartlett, 1959) content in some instances.

Phosphorus Analysis

For both the phosphorus and galactose determinations the lipid spots were detected with iodine vapor, the areas outlined and the iodine allowed to vaporize. Phosphorus containing areas were scraped into pyrex test tubes. Prior lipid elution was not required since the highly purified silica gel HR does not interfere with the assay. To each tube 0.5 ml of 10 N sulfuric acid was added and the tubes were placed in a metal heating block at 150-160 C for 3 h. A few drops of 30% H_2O_2 were then added and the solution was heated an additional 1.5 h to complete combustion. To the cooled hydrolysate 4.6 ml of 0.22% ammonium molybdate and 0.2 ml of Fiske Subba Row reagent was added, the contents mixed thoroughly and heated for 7 min in a boiling water bath. The samples were centrifuged at 10,000g for 5 min and the optical density of the clear solution read to 830 nm with a Beckmann Model 25 spectrophotometer. A representative absorption scan and standard curve is shown in Figure 3.

Galactose Analysis

Glycolipid spots were scraped into centrifuge tubes. The lipids were deacylated by the addition of 2 ml of 2 N H_2SO_4 to each tube and heating in a boiling water bath for 1 h, during which time they were shaken 4 to 5 times. The absorbent was then pelleted at 10,000g for 5 min and 1 ml aliquots were withdrawn from each tube. To each aliquot 1 ml of 2% phenol and 4 ml of concentrated H_2SO_4 were added followed by immediate mixing. After cooling for 20 min the absorbance was read at 490 nm. Representative absorption scans of galactose, galactolipids and sulfolipid are shown in Figure 4A. Galactose can be substituted for sulfoquinovose as a standard in the sulfolipid assay since equal weights of galactose and sulfoquinovse give the same net absorbance (Roughan and Batt, 1968). A representative standard galactose curve with standards treated the same way as the sample is shown in Figure 4B.

Internal Fatty Acid Standard

Lipid areas were visualized by spraying the plates with Rhodamine 6G (Allied Chemical). The spray solution was prepared just before use by mixing an equal volume of 0.006% aqueous Rhodamine 6G and 2N NaOH. Best results were obtained if the developed plates were dried under vacuum for 2 min. Immediately followng spraying the plates were viewed under short wave ultra violet illumination. The lipid areas (yellow-orange fluorescence) were outlined, and the spots were scraped into 15 ml screw-capped (with teflon liners) test tubes. The transesterification mixture containing 5 ml of 5% H_2SO_4 in methanol and an accurately measured quantity of the internal standard was then added. For the major lipids: monogalactosyl diglyceride (MG), digalactosyl diglyceride (DG), phosphatidyl choline (PC) one ml containing 10 ug of heptadecanoic acid (C:17) (Applied Science) in methanol gave adequate detector response. However, for the lipids present in lower amounts: phosphatidyl ethenolamine (PE), sulfolipid (SL) and phosphatidyl inositol (PI) 0.5 ml containing 5 ug of the internal standard in methanol was added. The screw caps were loosely closed during the initial 10 min of heating in an oven at 70 C. They were then tightened securely and heated for 2 h at 70 C. The contents were allowed to cool, then diluted with 5 ml of distilled water and the methyl esters were extracted with 3 successive 2 ml portions of hexanes (bp 65.2 to 67.6 C). Pasteur pipettes were used to collect the hexane phase which separates quickly following mixing. The pooled extracts for each sample were concentrated to near dryness under nitrogen, transferred with several washings to 1 ml vials and concentrated to dryness under nitrogen. The methyl esters were resolubilized in 50 ul methanol for the major lipids and 25 ul for those present in lower amounts. The vials were capped and stored under nitrogen at -20 C until required.

The fatty acid methyl esters were analyzed on an Aerograph model 200 gas chromatograph equipped with a hydrogen flame ionizing detector. A coiled stainless steel column (1.7 m x 3 mm) packed with 10% ethylene glycol adipate on Anakrom SD, 90/100 mesh (Analabs) was used with nitrogen as carrier. The column temperature was programmed at 8 C /min from 100 to 195 C with the injector temperature held constant at 240 C. A 2 to 4 ul sample prepared as described above gave good detector response.

For quantification of the methylesters and in turn their respective acyl lipids the ester peak areas were compared to that of the C:17 internal standard. The response factors were determined with quantitative fatty acid methylester mixtures (H103 and K108, Applied Science) and are given in Table II. Thus for an individual lipid spot collected from a thin layer plate the lipid was quantified according to:

nanomoles lipid = nanomoles standard (sum of adjusted ester peak areas/peak area of standard) x 1/n.

where n = 2, the number of acyl residues in the lipid.

Table II. Response factors of the flame ionizing detector for fatty acid methyl esters.

Methyl Ester	by Weight	Molar[1]
16:0	1.03	0.97
17:0	1.00	1.00
18:0	0.99	1.04
18:1	1.11	1.16
18:2	1.09	1.13
18:3	0.98	1.01

ratios are averages of three determinations.
[1] peak areas were multiplied by these factors to convert to molar ratios.

G. Chlorophyll Determination

A 2 ml aliquot of the C:M extract was concentrated to dryness under dim light and resolubilized in 80% acetone. The chlorophyll was determined according to Arnon (1949). The revised coefficients of Jeffery et al. (1974) were used to quantify the chlorophylls separated by thin layer chromatography.

H. Nitrogen Analysis

The nitrogen content of the pigment free residue was determined using a micro Kjeldahl digest and the ammonia was quantified by the phenol-hypochlorite procedure (Jacobs, 1965; Mitchell, 1971). A representative absorption scan and standard curve are shown in Figure 5. Nitrogen values were converted to protein content using the factor 6.25.

I. Gramine Analysis

Gramine content was estimated by the xanthydrol method for tryptophan analysis (Dickman and Crockett, 1956; Moore et al., 1967; Woods and Clark, 1971). Standards and sample were located on the TLC plates by spraying lightly with the xanthydrol solution (0.1% xanthydrol in 95% ethanol and 5% conc HCl). The spots were scraped into centrifuge tubes and the silica gel HR was extracted with 2 successive 2 ml portions of 6 N HCl. To the pooled supernatants 1 ml of xanthydrol reagent, containing 1 mg xanthydrol in acetic acid, was added and the contents were heated in a boiling water bath for 15 min. After cooling, 1 ml of sodium bisulfite, 1.5 mg/ml, was added. The absorbance at 500 nm was read after 30 min. A representative standard curve and absorption spectrum is shown in Figure 6.

To ensure adequate sample for infra red spectral analysis gramine was isolated from both normal and mutant barley leaves using the method of Schneider _et al._, (1972). The gramine fraction was subjected to the 2D TLC system as outlined for lipid separations except isopropylamine was not included in the second solvent system. The gramine area was visualized by exposure to iodine vapor and eluted from silica gel with C:M (1:1; v/v). The clear solution was

Figure 3. Absorption scan of the phosphomolybdate complex (A) and a typical standard curve used for phosphorus estimations (B).

Figure 4. Absorption scans of galactose and sugar constituents of monogalactosyl diglyceride (MG), digalactosyl diglyceride (DG) and sulfoquinovosyl diglyceride (SL) from barley leaves estimated by the phenol-H_2SO_4 procedure (A) and a typical standard curve used for galactose estimations (B).

Figure 5. Absorption scan of the ammonium complex estimated by the phenol-hypochlorite procedure (A) and a typical standard curve used for nitrogen estimations (B). $(NH_4)_2SO_4$ was used as the standard.

Figure 6. Absorption scan of the gramine-xanthydrol complex (A) and a representative standard curve used for gramine estimations (B). The gramine content was quantified using the xanthydrol method.

Figure 3

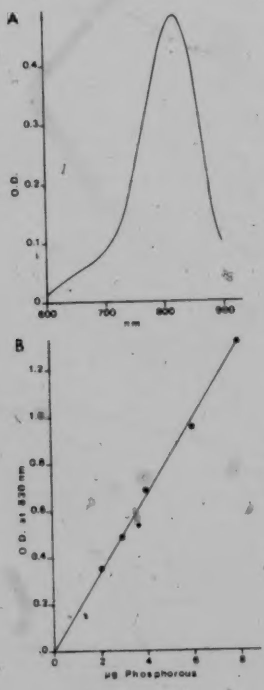


Figure 4

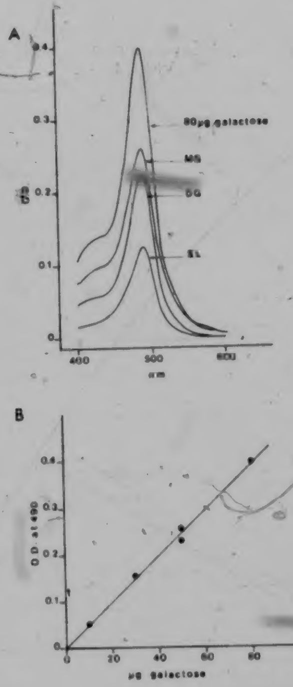


Figure 5

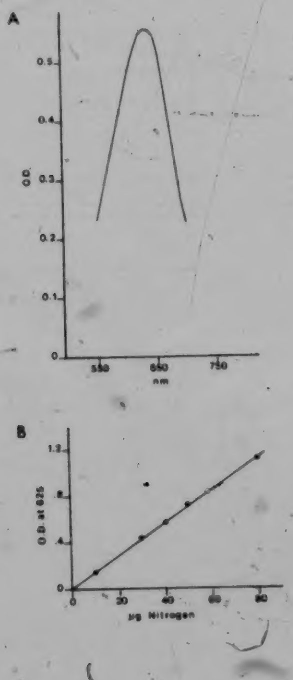
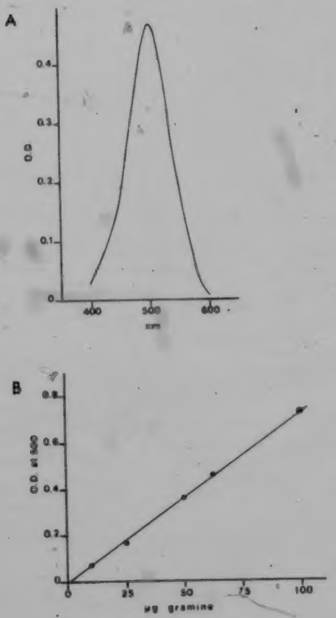


Figure 6



concentrated to dryness and resolubilized in a small volume of carbon tetrachloride. The IR spectrum was determined with a Perkin Elmer 421 grating spectrophotometer (Spectral Laboratories, Department of Chemistry, University of Alberta). Spectral analysis was attempted on gramine extracted with the total lipid extract and further separated by 2D TLC, however the sample was not sufficient even after pooling several plates.

J. Analysis of Pigment Remaining on the Sephadex Column

Pigments remaining on the G-25 column were completely eluted with 25 ml of distilled water. The eluent was concentrated by freeze drying and redissolved in a small volume of water. Aliquots were applied to silica gel HR TLC plates and screened according to the procedures outlined by Egger (1969). The pigmented compounds did not move from the origin in the solvent systems: A, benzene:acetone (9:1 v/v) which would separate slightly polar phenolic derivatives, and B, chloroform:acetic acid:water (50:45:5,v/v) which would separate phenolics of medium polarity. Separations were obtained with solvent system C, ethyl acetate:-butanone:formic acid:water (50:30:10:10, v/v) developed for strongly polar plant phenolic derivatives including flavone-glycosides and anthocyanidins.

The extract was further studied by one and two dimensional paper chromatography as well as visible and ultraviolet spectral analysis. The upper phase of the butanol:acetic acid:water (4:1:5 v/v mixture was employed as the solvent system in the one dimensional study (Egger, 1969). Two dimensional ascending chromatography was performed by folding a Whatmann #1 sheet over a 20 x 20 cm glass plate with the paper secured at the top of the plate for each dimension. The solvent systems were; I tertiary butanol:acetic acid:water (3:1:1 v/v) and II acetic acid:water (15:85 v/v) (Mabry et al., 1970). Development time was 12 h in the first and 45 min in the second direction. The chromatograms were viewed under UV light and the fluorescent zones outlined. For UV and visible spectral analysis the zones were eluted with ethanol containing 30% water. Reaction with sodium ethoxide solution consisted of adding a sodium hydroxide pellet to the sample cuvette containing the aqueous ethanol extract, swirling briefly, withdrawing the pellet and rescanning.

K. Carotenoid Estimation

Carotenoids were extracted, purified and quantified essentially by the procedures outlined by Jeffrey et al. (1974) and Jeffrey (1968). All procedures were carried out under dim light.

Extraction

The barley leaf segments were initially extracted with 80% acetone, however a reddish pigment remained in the residue. Further extraction of the residue with C:M (2:1, v/v) followed by 2D TLC and visible spectral analysis, by the procedures described below, showed the pigment consisted mainly of β-carotene. Traces of chlorophyll a and b were also evident. Therefore samples for quantitative carotenoid determinations were initially extracted with C:M 2:1 (v/v), concentrated to dryness on a flash evaporator with the vacuum released under nitrogen and resuspended in 80% acetone. The pigments were immediately transferred to diethyl ether by adding an equal volume of diethyl ether to the acetone resolubilized material, shaking with a volume of 10% NaCl at least 10 times that of the acetone volume and placed in a deep freeze at -20 C for 15 min. This was performed in 100 ml graduated cylinders. The ether phase containing the pigments separated to the top and was collected using a Pasteur pipette and the interface was washed with 2 successive 2 ml portions of ether. The combined extracts were concentrated to dryness under nitrogen and the pigments resolubilized and made to volume in ligroine. A few crystals of NaCl were then added to remove traces of water and the contents were stored briefly (1 h) under nitrogen at -20 C.

Thin Layer Chromatography

The pigments were separated by 2D TLC on powdered sucrose plates. Powdered sucrose (icing sugar, Canadian Sugar Factories, Lethbridge, Alberta) was passed through a 200 mesh (74 micron aperture) screen, dried at 90 C for 1 h and cooled in a desiccator. Thirty grams of sucrose were shaken with 50 ml of chloroform:ligroine (1:1 v/v) in a glass stoppered erlenmeyer and applied as a 250 um layer to 20 x 20 cm glass plates using a Quick-fit apparatus (Quick Fit Instruments, England). The whole apparatus was placed in a fume hood and the plates allowed to dry for 15 min before storing over silica gel in a desiccator.

Good separations were obtained with up to 5 ug of total pigment applied to the plate. To obtain adequate sample for quantitative analysis the pooled contents from 4 plates were required. Thus the sample size and volumes used were approximately 1 gm fresh weight of leaf segments for the mutant and 0.5 gm for the normal. The C:M extract was resolubilized in 7 ml of 80% acetone, 7 ml of diethyl ether were added and the total volume was brought to 100 ml with cold 10% NaCl. The pigments were brought to volume with ligroine, 1 ml for the mutant and 2 ml for the normal and 10 ul were spotted per plate. The sample was applied under a stream of nitrogen and the chromatography tanks prepared as described for lipid analysis. Solvent systems for the first and second dimensions were 2% n-propanol in ligroine and 40% chloroform in ligroine respectively. The corresponding developing times were approximately 12 min and 8 min.

The pigment spots were scraped into centrifuge tubes and eluted with 2 successive 2 ml portions of the respective solvents listed below. The contents were concentrated to volume and the absorption spectra recorded on a Beckman model 25 spectrophotometer.

The solvents and extinction coefficients (E^{1}_{1}%cm) at the indicated wavelengths (nm) were chlorophyll a (acetone) E_{663}= 881.5; chlorophyll b (acetone) E_{645} = 505.9; β-carotene (hexanes) E_{450} = 2505; lutein (ethanol) E_{447} = 2550; violaxanthin (ethanol) E_{441} = 2550; neoxanthin (ethanol) E_{438} = 2270 (Jeffrey et al., 1974).

IV. RESULTS

A. Lipid Extractions

A comparison of the three procedures frequently used for the extraction and purification of lipids is shown in Table III.

Table III. Gravimetric determination of the total lipid content of barley leaf segments using three extraction procedures. Results are for the apical 5cm leaf segments of 6 day old mutant barley seedlings.[1]

Isolation Procedure	mg/g fresh wt
Folch *et al* (1957)	11.14 11.19
Bligh and Dyer (1959)	8.95 9.29
Williams and Merrilees (1970)	11.00 10.83

[1] values are duplicate determinations from single extractions.

The Folch *et al*. (1957), Bligh and Dyer (1959) and Williams and Merrilees (1970) methods gave similar total lipid estimates. The three extraction procedures also gave similar patterns for the polar lipids on 2D TLC with the solvent systems of Allen and Good (1971) as well as those of Nichols (1964) (data not shown). The Williams and Merrilees method was selected for further analysis because the removal of the water soluble constituents with Sephadex was preferable to the phase separation procedure of Folch *et al.* (1957)

B. Flavonoids

Separation profiles of the aqueous eluent from the Sephadex G-25 column by paper chromatography are shown in Figures 7 and 8. The indicated color patterns evident when viewed under UV light are characteristic of various flavanoid classes (Swain, 1976). The spectral scans of zones 3, 4 and 5 eluted from the paper chromatogram (Figure 7) are characteristic of flavones and flavonols (Jurd, 1963; Markham and Mabry, 1975). The spectral scan of zone 4 shown in Figure 9A and the shift in absorption maximum on reaction with sodium ethoxide (Figure 9B) was also characteristic of zones 3 and 5. The spectral scans of the other zones were different. The 340 nm peak of zone 6 was a minor shoulder, however a shift to 380 nm occurred on treatment with sodium ethoxide. The sample in zone 2 was not sufficient and zone 1 (origin) displayed one peak in the 270 to 280nm range. Comparison of the two dimensional paper chromatogram (Figure 8) with the separation patterns obtained by Mabry *et al.* (1970) suggested that flavonol and flavone glycosides were present. Since flavonoids exist mainly as glycosides in nature (Swain, 1976) these results tentatively suggest the pigments remaining on the column are flavonoids and not lipid breakdown products.

C. Gramine

An outline of the total leaf lipids separated by 2D TLC is shown in Figure 10. The area identified as gramine has not been, to the author's knowledge, identified in leaf lipid studies. Charring with 50% sulfuric acid readily outlined all areas as shown in Figure 10 except gramine, which developed a rose color upon standing overnight. The gramine area was readily outlined with iodine vapors and was visible as a dull reddish color on plates sprayed with Rhodamine 6G and viewed under UV. Gas liquid chromatography (GLC) analysis revealed the absence of fatty acids in the C16 to C18 range. The area was not detected using identical extraction procedures with spinach leaves and with rye seedlings (Thomson and Zalik, 1973), suggesting the unknown was specific to barley. The unknown cochromatographed with authentic gramine (K and K Laboratories) and also with the gramine fraction prepared from barley leaves according to the procedure of Schneider _et al_. (1972). The gramine area also gave a positive reaction with Van Urk reagent spray for indoles (Kaldeway, 1969). The infrared spectrum of both authentic gramine and the unknown were very similar as shown in Figure 11. Thus the evidence suggest the unknown is gramine or a closely related indole compound.

Recovery of authentic gramine from the Sephadex G-25 column was estimated at 85% suggesting that the leaf gramine content could be quantified from the C:M extract. As shown in Table IV, the gramine content of the mutant was

Table IV. Gramine content of the apical 3cm leaf segments of 4 and 7 day old mutant and normal barley seedlings.

	ug/g fresh wt.	
days	**Mutant**	**Normal**
4[1]	756±34	1083±17
7[2]	1000	1566

[1] mean±SE of 4 determinations.

[2] single determinations.

approximately two thirds that of the normal and increased in both seedling types as the leaves matured.

D. Lipid Identification

The areas outlined in Figure 10 are representative of both mutant and normal seedlings at the growth stages analyzed. PI, PC, PE and PG gave the characteristic blue color specific for phospholipids when sprayed with molybdenum blue reagent (Dittmer and Lester, 1964). PE developed the red-violet color characteristic of free amino groups when sprayed with ninhydrin reagent (Skipski and Barclay, 1969) and PC gave the orange color characteristic of choline when sprayed with Dragendorff's reagent (Skipski and Barclay, 1969). PI cochromatographed with authentic PI (plant source, Applied Science). Fatty acid analysis of PG from green leaf material revealed the presence of trans-3-hexadecanoic acid, unique to PG. Early color development during charring with 50% H_2SO_4 gave a purple

Figure 7. Outline of flavonoid-like compounds present in the chloroform:methanol (2:1, v/v) lipid extract from 6 day mutant barley leaves separated by single dimensional paper chromatography. The pigmented complexes remained in the Sephadex G-25 column following elution of the lipid fraction with chloroform. They were completely removed with water. The aqueous eluent was concentrated by freeze drying and separated by paper chromatography using a butanol:acetic acid:water (4:1:5, v/v upper phase) solvent system. The zones were visualized under uv. F-fluorescent.

Figure 8. Outline of flavonoid-like compounds present in the chloroform:methanol (2:1, v/v) lipid extract from 6 day mutant barley leaves separated by two dimensional paper chromatography. Isolation procedure is given in Figure 7. Solvent system contained tertiary butanol:acetic acid:water (3:1:1, v/v) in the first dimension and acetic acid:water (15:85, v/v) in the second dimension. The zones were visualized under uv. f-faint; F-fluorescent; B-blue; Y-yellow.

Figure 9. Spectral scans of flavonoid-like compounds present in the chloroform:methanol (2:1, v/v) lipid extract from 6 day mutant barley leaves. Scanned between 200 and 500 nm.

A. Absorption spectrum of zone 4 (Figure 7) in ethanol.

B. Absorption spectrum of zone 4 (Figure 7) in ethanol made alkaline with sodium hydroxide pellets.

Figure 7

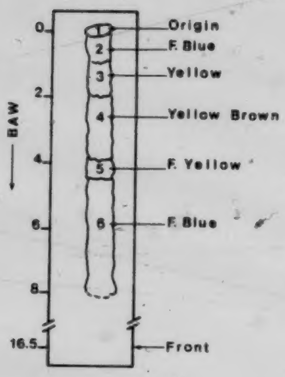


Figure 8

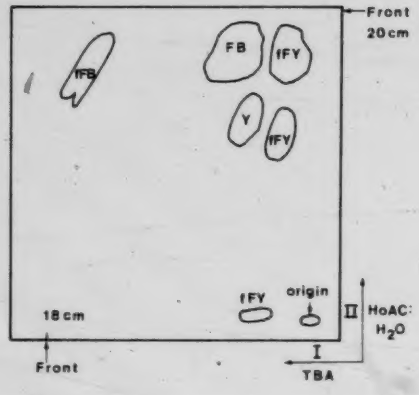


Figure 9

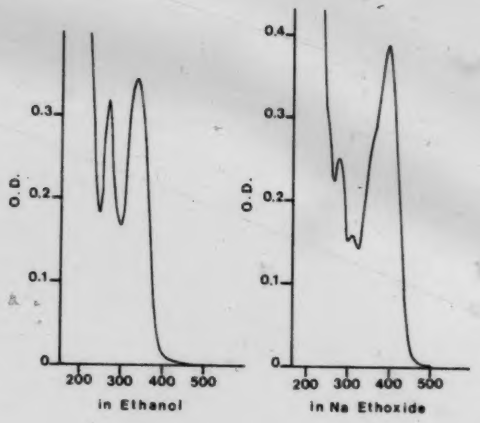


Figure 10. Outline of a representative two-dimensional thin layer chromatogram of the polar lipids isolated from leaf segments of 4, 6 and 8 day mutant and normal barley seedlings. The silica gel HR thin layer plate was developed in the first dimension with chloroform:methanol:water (65:25:4, v/v) and in the second dimension with chloroform:methanol:isopropylamine:ammonium hydroxide (65:35:0.5:5, v/v). The zones were identified as outlined. PI - phosphatidyl inositol; PC - phosphatidyl choline; PG - phosphatidyl glycerol; PE - phosphatidyl ethanolamine; SL - sulfolipid; DG - digalactosyl diglyceride; MG - monogalactosyl diglyceride.

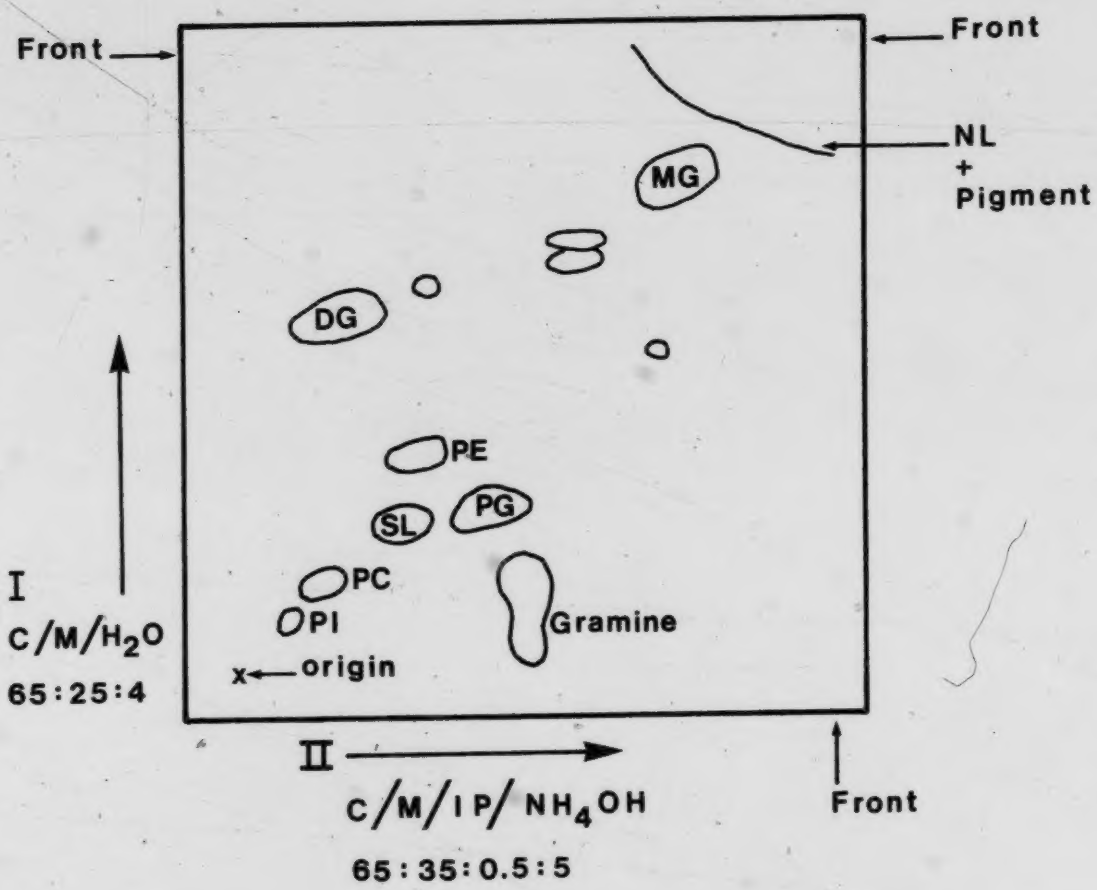
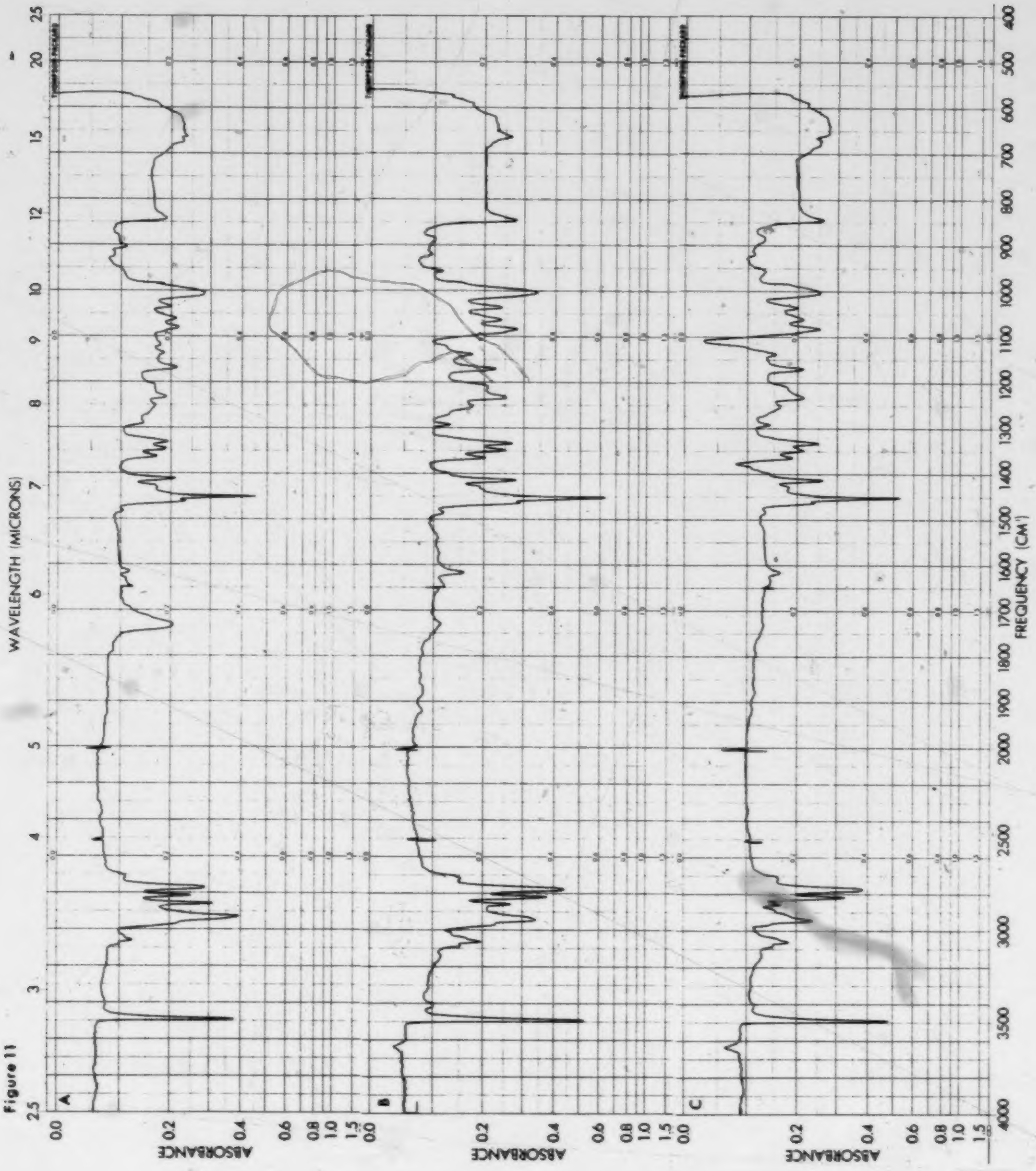

Front
Front
NL
+
Pigment
MG
DG
PE
SL
PG
PC
PI
Gramine
x origin
I
C/M/H_2O
65:25:4
II
C/M/IP/ NH_4OH
65:35:0.5:5
Front

Figure 11. Infra-red spectra of authentic gramine (A) and gramine isolated from 8 day mutant (B) and 8 day normal (C) barley leaves. Gramine was isolated from both mutant and normal barley leaves using the method of Schneider _et_ al (1972). Authentic gramine as well as plant isolates were subjected to the 2D TLC system as outlined in Figure 10 for the lipid separations, except isopropylamine was not included in the second solvent system. The gramine areas were visualized by exposure to iodine vapors and eluted from the silica gel with chloroform:methanol (2:1, v/v) and concentrated to dryness. The sample was resolubilized in carbon tetrachloride and the spectrum determined with a Perkin Elmer 421 grating spectrophotometer.

Figure 11


WAVELENGTH (MICRONS)
2.5
3
4
5
6
7
8
9
10
12
15
20
25
FREQUENCY (CM⁻¹)
4000
3500
3000
2500
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
A
B
C
ABSORBANCE
0.0
0.2
0.4
0.6
0.8
1.0
1.5

color characteristic of glycolipids and sterols of MG, DG and SL. The two spots on the diagonal below MG were also initially purple and are likely cerebrosides (Allen and Good, 1971). The fatty acid composition of MG, DG and SL was characteristic of these lipids from green tissue (Heinz, 1977; Leech *et al.*, 1973; Mudd and Garcia, 1975; Sellden and Selstam, 1976) and the 2D TLC separation patterns were similar to those reported for spinach by Allen and Good (1971) except for the presence of gramine.

E. Lipid Quantification

Quantification of the acyl lipid constituents by analysis of either the polar head groups or fatty acid moieties using C:17 as the internal standard gave results that were in close agreement as shown in Table V. These results suggest minimal comigration of phospholipid and galactolipid classes and show good agreement for the two methods. Recovery of lipids from the TLC plates estimated by phosphorus analysis ranged from 77 to 86% with an average of 83% for 4 determinations.

F. Changes in Lipid Constituents

The protein content of the residue insoluble in lipid solvents and the total lipid content of leaf segments harvested at 4, 6, and 8 days is shown in Figure 12. The seedlings of the normal and mutant were similar in leaf dimensions and fresh weight per leaf. The normal contained

Table V. Estimation of individual lipid constituents by either phosphorus or galactose analysis and with the C:17 internal standard.

	nmoles /g fresh wt						
	PI	PC	PE	PG	MG	DG	SL
mutant 6 day[1]							
Head Group[2]	208.1	1209	571.6	244.2	1366	1082	304.8
Acyl Group[3]	182.9	1181	539.0	238.2	1807	1081	330.0
Mutant 8 Day[1]							
Head Group[2]	228.7	1202	559.0	265.9	2059	1141	--
Acyl Group[3]	165.0	1052	500.2	200.3	1913	1244	361.6
Normal 6 Day[1]							
Head Group[2]	233.8	1390	461.4	529.1	3418	3111	--
Acyl Group[3]	249.0	1482	473.0	466.8	3848	2530	697.5
Normal 8 Day[1]							
Head Group[2]	272.2	1394	488.8	444.4	3874	2999	--
Acyl Group[3]	249.5	1225	509.1	497.1	3750	2761	514.2

[1] aliquots are from the same total lipid extract of apical 3cm leaf segments.

[2] galactose and phosphorus estimates are averages of 2 determinations for the mutant and single determinations for the normal.

[3] single determinations.

consistently more lipid and protein over the growth stages. Increases were evident in the mutant throughout the growing period, however for the normal the increases were most marked between 4 and 6 days. A slight decrease occurred by 8 days. This decline was perhaps the result of the slight browning of the leaf tips in the normal by 8 days. The lipid content of the mutant averaged 67% of the normal.

Chlorophyll accumulation showed a sharp increase in the normal between 4 and 6 days with a leveling off by 8 days. The increase in the mutant was most pronounced between 6 and 8 days (Figure 13). The chlorophyll content of the mutant increased from 7 to 50% relative to the normal.

Marked increases in the major chloroplast acyl lipids, MG and DG (Figure 14 A, B) also occurred at the growth stages where chlorophyll accumulation was most pronounced. These data represent a different experiment than those in Table V and show a larger increase in the major galactolipids, however similar trends were evident in the two experiments. The MG:DG ratio was 1.6, 1.8 and 1.6 at 4, 6 and 8 days for the normal and 1.2, 1.6 and 1.9 respectively for the mutant. The minor chloroplast lipids PG and SL showed a slight increase or little change with increasing age. The major lipids of the non-chloroplast membranes, PC and PE, (Mazliak, 1977; Mudd and Garcia, 1975) decreased throughout the development period. The decrease was most evident for the PC content (Figure 14 A, B). The mutant contains proportionally more of the major acyl lipids of the non-chloroplast membranes, PE and PC at 4 days (Figure 15). However in both plant types the major galactolipids MG and DG accounted for approximately 45 and 25% respectively of the total acyl lipids by 8 days (Figure 15).

The adjacent leaf segments between 3 and 6 cm from the apical tip were lower in galactolipid content than the corresponding more mature apical segments (Figure 16A, B). Large increases occurred as the segments matured. The major non-chloroplast membrane lipids, PC and PE, contents were however similar on a fresh weight basis to those of the apical 3 cm leaf segments for both the normal and mutant (Figure 16 A, B).

G. Variations in Fatty Acid Composition of Acyl Lipids

The fatty acid composition of the acyl lipids, except for PG was qualitatively similar, however the proportions of the representative fatty acids present were unique to the individual lipid classes (Figures 17-23). MG in both plant types was highly unsaturated with linolenic acid (18:3) contributing approximately 90% of the fatty acid content (Figure 17). DG was also highly unsaturated, with 18:3 representing close to 80% (Figure 18). A distinguishing feature between the two galactolipids was the higher percentage of palmitic acid (16:0) in the DG representing close to 18% whereas in MG 16:0 was present in only trace amounts. Palmitic and linolenic were the major acyl moieties of sulfolipid representing 35 and 60% respectively (Figure 19). Trans-3-hexadecanoic acid (16:1-3t) was unique to PG, initially representing approximately 5% in both plant types at 4 days and increasing to 25-30% by 8 days (Figure 20). A corresponding decrease from approximately 40 to 20% occurred in palmitic acid. Linolenic acid was also a major constituent of PG representing 35 to 40%. The major

Figure 12. Protein and lipid content of the apical 3 cm leaf segments of 4, 6 and 8 day mutant and normal barley seedlings. After chloroform:methanol (2:1, v/v) extraction protein in the residue was estimated using a micro Kjeldahl digest. Total lipid content was estimated gravimetrically.

Figure 13. Chlorophyll content of the apical 3 cm leaf segments of 4, 6 and 8 day mutant and normal barley seedlings. An aliquot of the chloroform:methanol extract (2:1, v/v) was concentrated to dryness, resolubilized in 80% acetone and chlorophyll was estimated according to Arnon (1949).

Figure 12

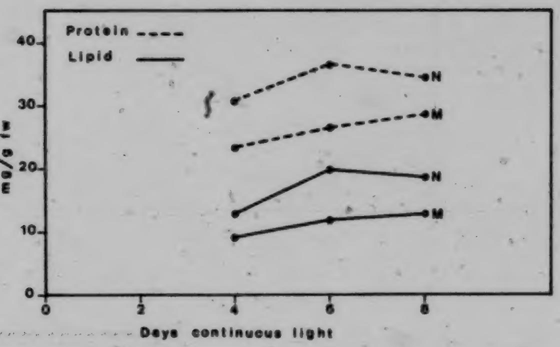


Figure 13

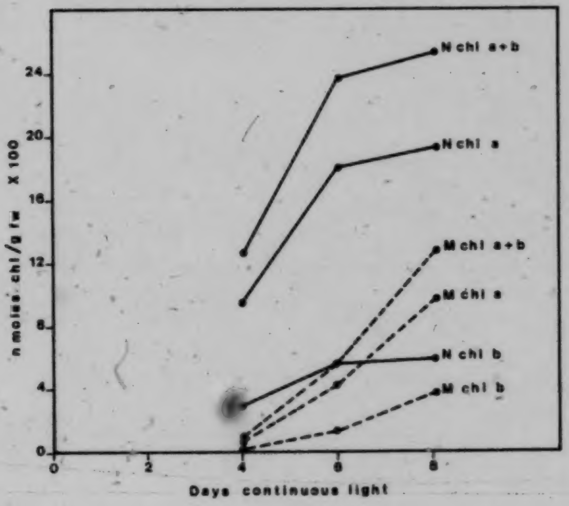


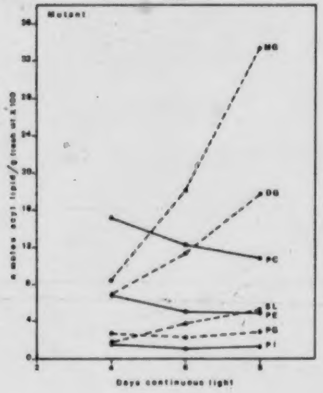
Figure 14. Acyl lipid content of the apical 3 cm leaf segments of 4, 6 and 8 day mutant (A) and normal (B) barley seedlings. The lipids were separated by 2D TLC as outlined in Figure 10 and quantified using the C:17 internal standard as described in materials and methods. Data represent single determinations.

Figure 15. Acyl lipid content expressed as a proportion of total acyl lipid content for the apical 3 cm leaf segments of 4, 6 and 8 day old mutant and normal barley seedlings. Data calculated from Figure 14.

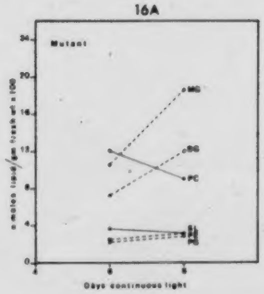
Figure 16. Acyl lipid content of the second 3 cm leaf segments of 6 and 8 day old mutant (A) and normal (B) barley seedlings. Quantification was as outlined in Figure 14.

Figure

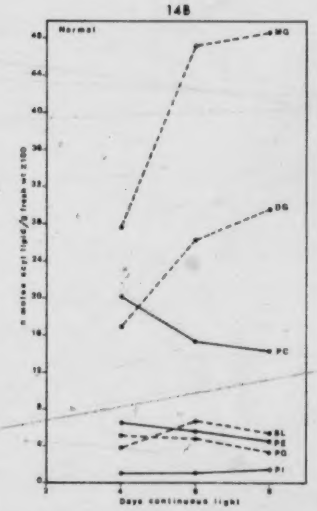
14A



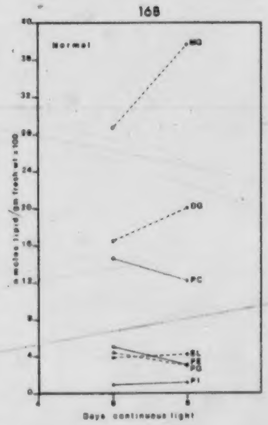
16A



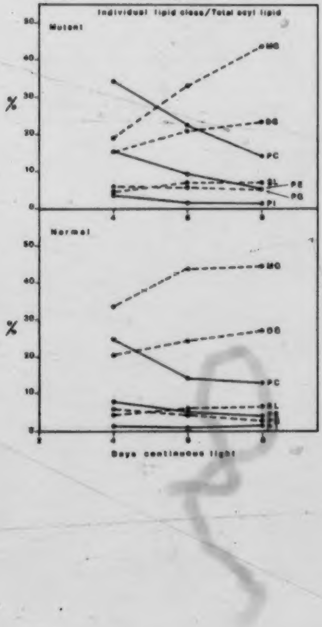
14B



16B

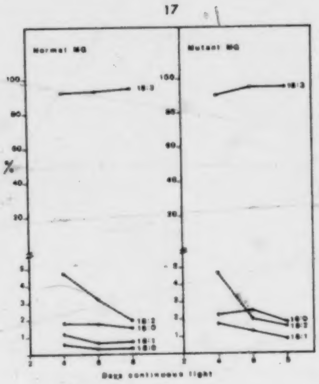


15

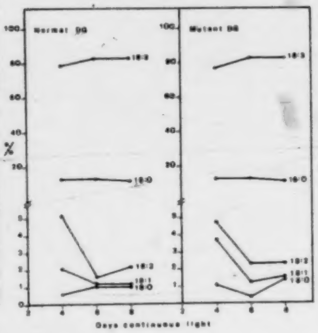


Figures 17-23. Changes in the fatty acid composition of acyl lipids from apical 3 cm leaf segments of 4, 6 and 8 day old mutant and normal barley seedlings. The acyl lipids were isolated and fatty acids quantified as outlined in Figure 10. Figure 21 (normal b and mutant b) are from the second 3 cm leaf segment.

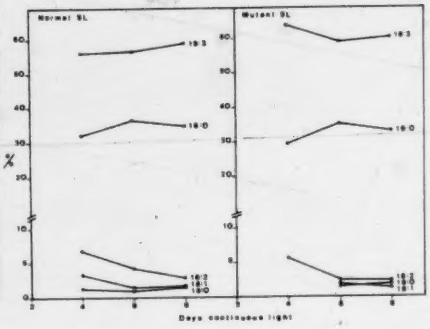
17



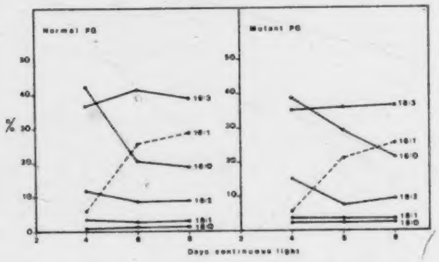
18



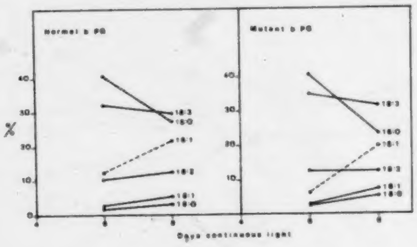
19



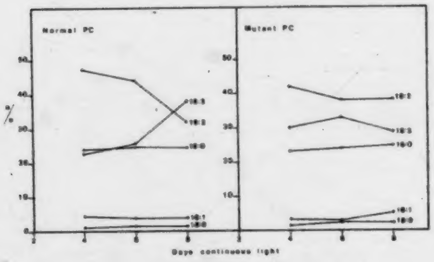
20



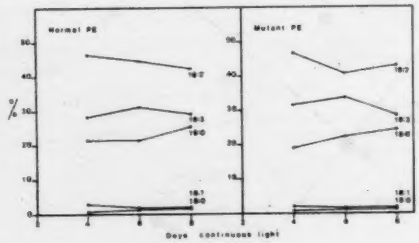
21



22



23



non-chloroplast lipids were more saturated, linoleic acid (18:2) represented 40 to 50% in both PC and PE (Figure 22 and 23) except for PC of the normal at 8 day in which case 18:3 became the major constituent at 38% (Figure 22). Linolenic acid otherwise comprised 25 to 35% and palmitic acid 20 to 25% of the fatty acids for both these lipid classes.

For all the lipid classes only minor quantitative fatty acid differences were evident between the normal and the mutant and minor quantitative variations occurred as the seedlings matured, except as indicated for trans-3-hexadecanoic acid in PG. The trans-3-hexadecanoic acid content of PG from 6 day mutant seedlings sampled from the second 3 cm leaf segment was present at approximately half (6%) the level present in the normal (12%) (Figure 21). The level in both plant types was approximately the same by 8 days, however the quantity was less than the level obtained in the more mature leaf segments.

H. Carotenoids

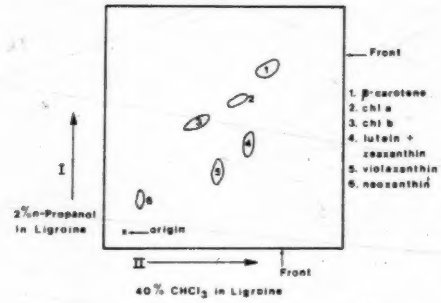
An outline of the leaf pigments separated on sucrose thin layer plates is shown in Figure 24. Although there was variation in the intensity and size of some pigment zones they were present in all growth stages of the mutant and the normal. α and β-carotene or their dihydroxy derivatives lutein and zeaxanthin are not resolved on sucrose plates with the solvent systems used (Jeffrey et al., 1974). These were taken as β-carotene or lutein for the work described. Pigments were identified by their similar migration patterns to those outlined by Jeffrey et al. (1974) with isolates from corn chloroplasts. Also the visible absorption spectra (Figures 25-29) and the position of the absorption peaks were characteristic of the identified carotenoids (Davies, 1976). Recoveries from the TLC plates estimated on a chlorophyll basis using the revised extinction coefficients of Jeffery et al. (1974) were greater than 92% except for 4 and 5 day mutant which averaged 75%. The low levels of the latter are perhaps due to the very low levels of chlorophyll in the mutant.

β-Carotene was the dominant carotenoid in the normal, increasing from approximately 50 to 80 ug/g fresh weight between days 4 to 6 (Figure 30). This represents 40 and 50% respectively, of the total carotenoids (Table VI). At days 4 and 5 lutein and violaxanthin were the major carotenoids of the mutant comprising approximately 30%, however by 6 days β-carotene became dominant increasing to 25 ug/g fresh weight by 8 days and representing 45% of the total carotenoid composition (Figure 30, Table VI). Lutein was the major xanthophyll in the normal throughout the development period, comprising 22 to 28% of the carotenoid content. This was followed by neoxanthin representing 16 to 21% and violaxanthin declining from 16% at 5 days to 8% at 8 days (Figure 30, Table VI). Similar levels of neoxanthin, violaxanthin and lutein accumulated in the mutant at 6 and 8

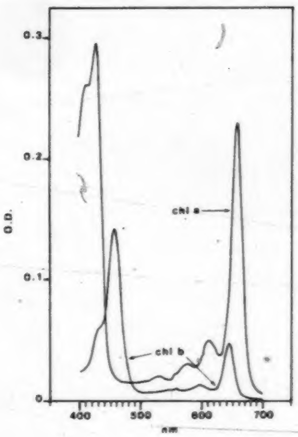
Figure 24. Outline of a representative two-dimensional thin-layer chromatogram of leaf pigments isolated from the apical 3 cm leaf segments of 4 to 8 day old mutant and normal barley seedlings. The leaf pigments were extracted and prepared for TLC as described in methods. The powdered sucrose plate was developed in the first dimension with 2% n-propanol in ligroine and in the second dimension with 40% chloroform in ligroine.

Figures 25-29. Absorption spectra of chlorophylls and carotenoids isolated from the apical 3 cm leaf segments of 7 day mutant barley seedlings and separated by 2D TLC on powdered sucrose plates. The pigments were eluted from the sucrose and scans taken in the respective solvents: chlorophyll a and b, acetone; β-carotene, hexanes; lutein, violaxanthin and neoxanthin, ethanol. These spectra were the same for the normal and mutant.

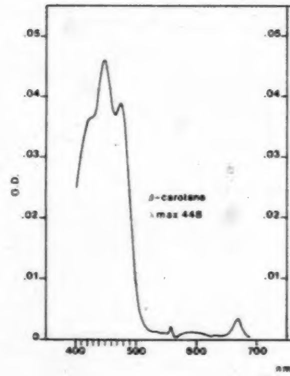
Figure 24



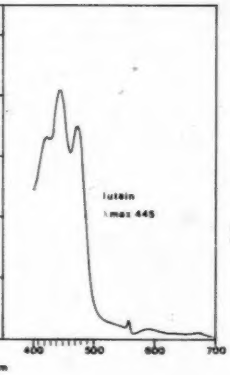
25



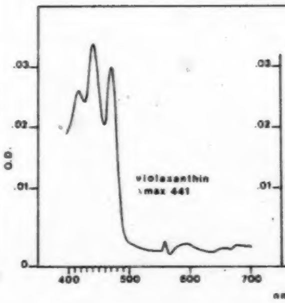
26



27



28



29

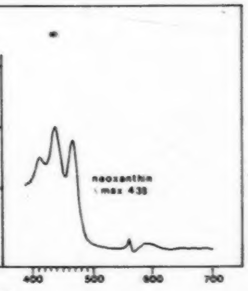
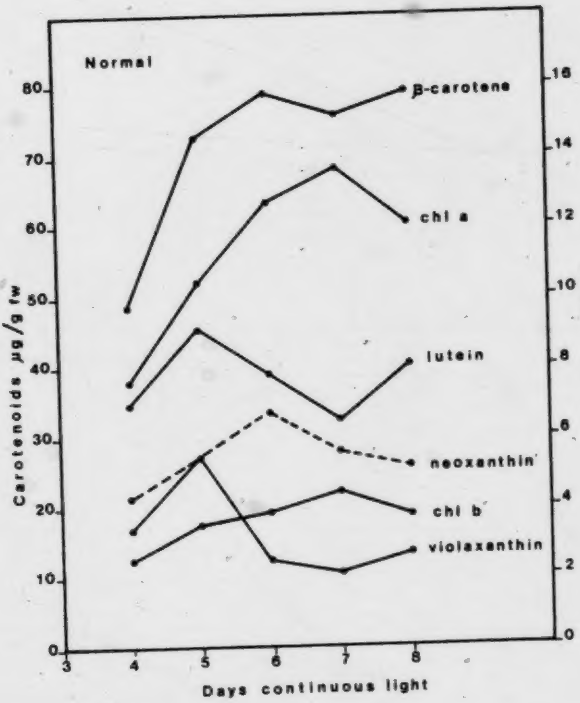


Figure 30. Chlorophyll and carotenoid content of the apical 3 cm leaf segments of normal (A) and mutant (B) barley seedlings sampled at daily intervals from 4 to 8 days. The pigments were separated by 2D TLC on powdered sucrose plates, eluted with the solvent systems as outlined in Figures 25-29 and quantified by their extinction coefficients (Jeffrey *et* al, 1974).


Normal
β-carotene
chl a
lutein
neoxanthin
chl b
violaxanthin
Carotenoids µg/g fw
80
70
60
50
40
30
20
10
0
Chl µg/g fw X 100
16
14
12
10
8
6
4
2
0
3
4
5
6
7
8
Days continuous light

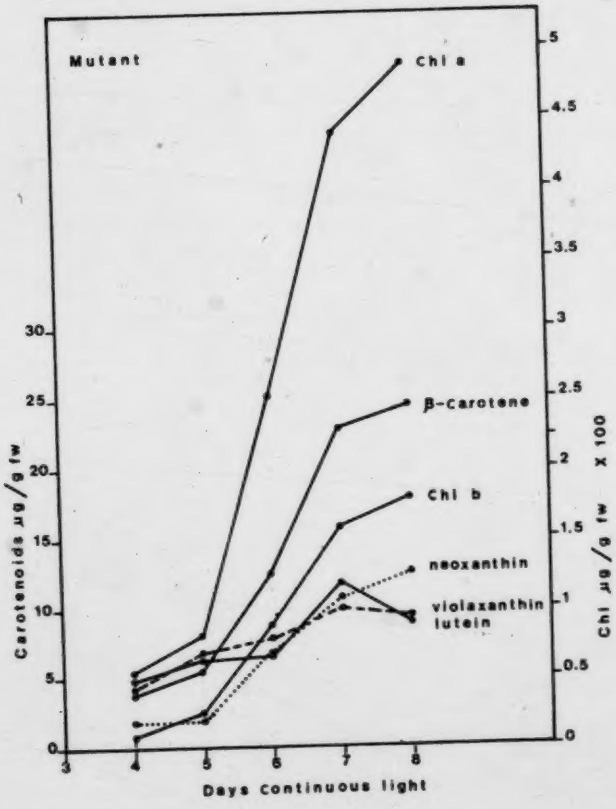

Mutant
Chl a
β-carotene
Chl b
neoxanthin
violaxanthin
lutein
Carotenoids µg/g fw
30
25
20
15
10
5
0
Chl µg/g fw X 100
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
3
4
5
6
7
8
Days continuous light

Table VI. Pigment ratios estimated from sucrose 2D TLC plates of pigment extracts from the apical 3cm leaf segments of normal and mutant barley seedlings harvested at daily intervals from 4 to 8 days[1].

	/Days	4	5	6	7	8
				Mutant		
chl a/b		5.16	3.35	2.84	2.76	2.73
carotenoid/chl		0.25	0.20	0.10	0.09	0.08
%	β-carotene	25	26	36	41	45
	lutein	32	30	19	21	16
	violaxanthin	30	33	23	18	15
	neoxanthin	13	10	21	19	23
				Normal		
chl a/b		2.98	2.86	3.24	3.11	3.15
carotenoid/chl		0.12	0.12	0.10	0.08	0.10
%	β-carotene	40	42	48	52	50
	lutein	28	26	23	22	25
	violaxanthin	14	16	8	7	8
	neoxanthin	17	16	21	19	16

[1] Data calculated from Figure 30.

days with each representing 15 to 23% of the total carotenoid content (Figure 30, Table VI). The lutein and neoxanthin content of the mutant was about 1/4 and 1/2 respectively to the levels reached in the normal. However, the ratio of carotenoids to total chlorophyll which was initially higher in the mutant at 25% was similar for both plant types by 6 days representing 10% of the total chlorophyll (Table VI). The chlorophyll a/b ratio also declined in the mutant during the 4 to 6 day interval approaching the value of 3 which is comparable to that of the normal (Table VI).

V. DISCUSSION

Since the chloroplasts are very active in cellular lipid biosynthesis and represent a rich source of cellular lipid which has an unique acyl lipid composition, a study of the lipid composition during the development of the mutant was undertaken to further characterize it. The separation patterns of the polar lipids (Figure 10) and leaf pigments (Figure 24) revealed all the acyl lipids and pigments of the mutant were qualitatively identical to the normal. Large quantitative differences however were evident (Figures 14 and 30). The contents of the major chloroplast acyl lipids and the chloroplast pigments were very low at 4 days in comparison with the normal at the same age. Previous studies on the mutant grown under near identical conditions have shown considerable differences in the young seedlings at 4 days in plastid size, plastid internal lamellar structure and lamellar protein composition (Jhamb and Zalik, 1973) and the photoreductive activities (Horak and Zalik, 1975) when comparisons were made to the normal barley of the same age. These differences were shown to be self-correcting with the plastid ultrastructure, lamellar protein content and photoreductive activities approaching those of the normal by 8 days. The mutant however still remained chlorophyll deficient at 8 days. Although one may speculate as to the cause of the lower chloroplast acyl lipid and pigment content in the mutant, the results perhaps best reflect a close coordination of the synthesis of macromolecules during plastid development. The absence or limiting amount of one component due to the nuclear mutation would likely affect the synthesis of several constituents. The results will be further discussed primarily from the developmental point of view.

The apical 3 cm leaf segments were selected for all the composition studies except where indicated (Figures 16 and 21). This was an attempt to select a more uniform plastid population for developmental studies. The leaf meristems of monocotyledons occur at the leaf base resulting in a linear array of cells with the most mature at the apical tip. Ultrastructural studies have shown a similar array in plastid maturity (Leech *et al.*, 1973; Robertson and Laetsch, 1974). The shoots were extended 3 cm by 4 days and the apical 3 cm segments were also selected for the 6 and 8 day analysis. The presence of less developed plastids in the younger leaf segments at 6 and 8 days is reflected in their acyl lipid and fatty acid composition (Figures 16 and 21).

Since most of the cellular constituents show considerable variation during the developmental study a suitable basis for comparison was sought. An increase was evident in the total lipid content and protein content of the residue insoluble in chloroform:methanol in both plant types (Figure 12). Both parameters likely reflect the state of chloroplast development since the chloroplasts have been shown to contain approximately 2/3 of the total leaf lipid in spinach leaves (Zill and Harmon, 1962) and approximately

75% of Oenothera leaf protein was contributed by the chloroplast fraction (Zucker and Stinson, 1962). Also the major chloroplast enzyme, ribulose bisphosphate carboxylase is estimated to contribute up to 50% of the total leaf soluble protein (Ellis, 1976). The increased protein content in this study is likely largely due to the increased RUBP carboxylase protein content which was shown to increase approximately 50% in the normal and 5 fold in the mutant between 4 and 8 days when the seedlings were grown under near identical conditions (Barankiewicz _et al._, 1979). Since the seedlings of the mutant and normal were of similar dimensions and fresh weight per leaf the comparison data were presented on a fresh weight basis. The results presented in Figures 12 and 13 are from the same sample preparation as those in Figures 14 and 16 so comparisons on protein content, total lipid content and pigment content can also be used.

The lipid constituents quantified (Figures 13 and 14, Table IV) represent 46, 53 and 65% of the total lipid content of the mutant and 60, 61 and 68% of the total lipid content of the normal at 4, 6 and 8 days respectively (Figure 12). The acyl lipids (Figure 14) represent 37, 41 and 48% of the total lipid content of the mutant and 50, 43 and 47% of the total lipid content of the normal at 4, 6 and 8 days respectively. The lipid constituents not analyzed were the neutral lipid fraction which migrated with the solvent front and the two spots on the diagonal below MG

(Figure 10). The most pronounced changes in the acyl lipid fraction between 4 and 8 days were the two major chloroplast lipids, monogalactosyl diglyceride (MG) and digalactosyl diglyceride (DG), increasing on a fresh weight comparison basis 4 fold and 2.5 fold respectively in the mutant and 1.8 fold for both MG and DG in the normal (Figure 14). This corresponded to an increase from 7 to 20% of the total lipid content for MG and 7 to 11% for DG in the mutant between 4 and 8 days. The corresponding values for the normal were for MG an increase from 16 to 20% and for DG an increase from 10 to 13% from 4 to 8 days. Several laboratories have shown that the lipid of the chloroplast fraction contains predominately monogalactosyl diglyceride and digalactosyl diglyceride and relatively low amounts of phospholipids (Kates, 1970; Mudd and Garcia, 1975; Leech and Murphy, 1976). The increased galactolipid content and corresponding decline in the major phospholipids, PC and PE (Figures 14;15) likely reflect an increased chloroplast membrane biosynthesis relative to other cellular membranes. Others have reported similar findings with greening leaves, showing the increase in galactolipids and decline in the PC and PE was most pronounced during the period of rapid chloroplast membrane formation and grana stacking (Bahl _et al_., 1976; Leech _et al_., 1973; Roughan and Boardman, 1972; Sellden and Selstem, 1976; Tremolieres and Lepage, 1971). The marked acyl lipid changes in this study also correlate with the formation of grana and increased photoreductive activity of the mutant (Horak and Zalik, 1975). The normal however, shows a slight decline in photoreductive activity between 4 and 8 days when activities are expressed on a lamellar protein basis (Horak and Zalik 1975) and during this interval the major chloroplast lipids still display a major increase (Figure 14). This may indicate that chloroplast membrane growth is still occurring in the normal between 4 and 6 days. The decline in activities on a lamellar protein basis may reflect the increased lamellar protein content due to an increase in the LHC a/b protein complex which is hypothesized to add in discrete units to the photosystem reaction centers (Arntzen, 1978). It is of interest that stroma lipids presumably containing acyl lipids have also been found to contribute to a significant proportion of the plastid lipid complement and are suggested to be lipoprotein complexes in transit between the different chloroplast membrane systems (Leech and Murphy, 1976; Poincelot, 1973).

The increase in the MG/DG ratio during the greening process has been reported by others and has been interpreted to reflect the increase in the relative contributions of the chloroplast lamellae and the chloroplast envelope to the galactolipid composition (Mudd and Garcia, 1975). Chloroplast envelope preparations from spinach and corn have a MG/DG ratio of 0.7 to 1 whereas the MG/DG ratio of the lamellae was 2.0 to 2.4 (Douce _et al_.,1973; Mackender and Leech, 1974; Poincelot, 1973). Analysis of stroma and lamellar preparations (Allen _et al_., 1972; Wintermans, 1971) and comparisons between mesophyll and bundle sheath chloroplasts (Bishop *et al.*, 1971) indicated the MG/DG ratios of the stroma and grana lamellae do not differ indicating the two galactolipids are distributed homogeneously in the chloroplast membrane.

The very low increase in the other characteristic chloroplast acyl lipids, PG and SL during greening has also been shown by others analyzing greening etiolated leaves (Behl *et al.*, 1976; Roughan and Boardman, 1972; Tremoliers and Lepage, 1971) and with whole tissue analysis of developing leaf segments (Leech *et al.*, 1973). Any increases in the PG and SL content are likely masked by the massive changes in the other cellular constituents during chloroplast development, since PG and SL contribute respectively approximately 6 and 5% of the total chloroplast membrane lipids (Leech and Murphy, 1976; Table 1). Increases, however were evident in studies performed on isolated plastids (Leese and Leech, 1976; Sellden and Selstam, 1976).

Although the individual acyl lipids all contain the same qualitative fatty acid composition, except for PG (Figures 20;21) which contained in addition 16:1-3t, the amounts of the individual fatty acids were unique to the lipid classes (Figures 17-23). This has been shown to be characteristic of lipids in green tissue (Mudd and Garcia, 1975; Harwood and Stumpf, 1976; Kates, 1970). The findings in this study are also in close agreement with those obtained for wheat and barley (Bahl et al., 1976; Sellden and Selstram, 1976) and show a higher level of unsaturation in PG and SL than found in maize, bean and pea (Leech et al., 1973; Roughan and Boardman, 1972; Tremolieres and Lepage, 1971). Very little change in fatty acid composition except for PG occurred over the developmental period sampled. This has been reported in greening studies of etiolated leaves. However, Leech et al. (1973) on sampling serial sections of developing maize leaves grown under natural light, under which they do not form etioplasts, found a marked increase in unsaturation as the plastids developed from the proplastid to mature plastid.

Trans-3-hexadecenoic acid (16:1-3t) increased markedly in both plant types between 4 an 8 days (Figures 20; 21). The fatty acid was specifically associated with PG. This is in agreement with other reports (Harwood and James, 1975; Leech and Murphy, 1976). The exact role of this unique fatty acid is not known. It is found specifically in photosynthetic tissue but does not appear essential for photosynthetic activity and grana stacking (For more complete discussion see pages 27-28).

The study of the carotenoid composition during the developmental period was also of interest in the further characterization of the virescens mutant. The same major carotenoids, β-carotene, lutein, violaxanthin, and neoxanthin have been found to be ubiquitous to all green tissue of higher plants and are suggested to be located exclusively in the chloroplast (Goodwin, 1976).

The virescens mutant was previously shown to be deficient in total carotenoid content, with the mutant attaining approximately half the content present in the normal by 7 days in glass house grown seedlings (Maclachlan, 1962). The present study represents an earlier sampling time as well as the quantification of the individual carotenoid constituents. The deficiency of the mutant was again evident, the total carotenoid content increased from 14% to 50% relative to the normal on a fresh weight comparison during the period of analysis. It is of interest to note the close correlation of the β-carotene concentration and that of chlorophyll a+b (correlation coefficient of 0.99) in the mutant sampled at daily intervals from 4 to 8 days. A close correlation was also evident in the normal between 4 and 6 days (r=+0.96). β-Carotene has been shown to be a constituent of both photosystem reaction centers and the LHC a/b complex (Thornber, 1975). A close correlation would thus be expected during the formation of the chloroplast internal membrane network. Keck et al. (1970) also describe a close correlation of the β-carotene and chlorophyll concentrations in 3 soybean genotypes of differing chlorophyll concentrations. The initial low β-carotene content relative to the total carotenoid concentration at 4 days in the mutant, which increases from 25 to 45% by 8 days (Table VI) likely reflects the increased concentration of carotenoids in the chloroplast lamella relative to chloroplast envelope.

The chloroplast envelope has been shown to be enriched in xanthophylls relative to β-carotene (Bishop, 1974; Douce *et al.*, 1973; Jeffrey *et al.*, 1974). The increased xanthophyll content in the mutant between 5 and 8 days (Figure 30) probably reflects the formation of the LHC a/b complex since the complex has been shown to contain all the carotenoids with β-carotene and lutein being the major carotenoids present (Thornber, 1975). This corresponds to the decrease in the chlorophyll a/b ratio (Table VI) which is suggested to be a marker for the formation of the LHC a/b complex (Arntzen, 1978). However little is known about the organization of carotenoids in higher plants. Some recent theories on the organization of carotenoids within the chloroplast membrane have been proposed. Anderson *et al.* (1978) indicate the excitation spectra which contribute to the chlorophyll a fluorescence emission demonstrate that carotenoids occur in all the recently isolated chlorophyll--protein complexes and further anticipate all the photosynthetic pigments of higher plants are complexed to protein. Rohmer *et al.* (1979) hypothesized that since chloroplasts do not contain significant amounts of sterols, carotenoids may serve to stabilize the membrane bilayer similar to the role proposed for polyterpenoids in prokaryotes. The incomplete extraction of β-carotene with 80% acetone but its complete removal with chloroform:-methanol (see Methods) perhaps indicates at least a fraction of the β-carotene is in close association with protein *in* vivo.

In summary acyl lipid and pigment compositional studies corroborate the previous findings that the virescens mutant displays a general lag in chloroplast development. The lag in lamellar protein formation and internal structure (Jhamb and Zalik, 1973), photoreductive activities (Horak and Zalik, 1975) and acyl lipid and pigment content suggest a close coordination of the synthesis of the chloroplast macromolecules.

Developmental studies on the virescens mutant are of interest because the lag in chloroplast development provides an alternative system to the commonly studied greening of etiolated tissue and 'static' mutations which are restricted in their degree of development. The very marked increase in MG and DG during the formation of the chloroplast internal structure, which showed a close correlation with the increase in chlorophyll content would suggest these lipids are the major membrane matrix lipids. The minor increases in the other characteristic chloroplast lipids perhaps reflect a more specialized role, perhaps as boundary lipids. These roles have been proposed from studies in which membrane preparations were subjected to to selective lipase digests and the resulting ultrastructural and enzyme activity changes monitored (see pages 22-24). The increased MG/DG ratio and increased β-carotene content relative to the total carotenoid content during the build-up of the chloroplast internal membrane network corroborates the findings based on fractionation studies which show differing ratios of these constitutents between the chloroplast envelope and internal lamellae.

Even though the chloroplast displays an unique acyl lipid content, a specific role of these constituents has not been described (Harwood and Stumpf, 1976). Since the chloroplast lipids are ubiquitous to higher plants Harwood and Stumpf (1976) suggested the absence of one or more of the lipids would probably be lethal. Developmental studies have not shown specific roles for individual acyl lipids since the full complement of acyl lipids is present in the undeveloped plastid. Reconstitution studies may serve as an alternative system to elucidate specific roles for them, but the limitations of this approach have been recently outlined by Sandermann (1978). Studies in which the membrane lipid composition has been altered *in vivo* are also of interest. Waring *et al.* (1976) reported that the phospholipid composition of etiolated tomato seedlings could be modified *in vivo* by treatment with ethanolamine and Tween-oleate.

VI. ACETYL CoA CARBOXYLASE ACTIVITY IN BARLEY SEEDLINGS

MATERIALS AND METHODS

A. Chemicals

S-acetyl coenzyme A (Lithium salt); alumina Cγ; ATP-disodium grade I; avidin type III, 10 units/mg protein; d-biotin; bovine serum albumin (BSA), fraction V; DL-dithiothreitol (DTT); 2(4-hydroxyazobenzene) benzoic acid (HABA); and protease type VI were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Ammonium sulfate (special enzyme grade) was obtained from Schwarz/Mann, Orangeburg New York, U.S.A.; hydroxylapatite powder, Bio-Gel HTP, from Bio-Rad Laboratories (Canada) Mississauga, Ontario; and ^{14}C sodium bicarbonate from New England Nuclear (Canada) Dorval, Quebec. The silica gel impregnated glass fiber sheets (Gelman Chromatography media ITLC-SG Type 20 x 20 cm) were obtained from Gelman Instrument Company, Ann Arbor, Michigan, U.S.A.

B. Buffers and Reagents

The pH of all buffers was adjusted at 20 C. Buffers containing dithiothreitol were prepared in bulk without DTT. The DTT was added fresh daily to new portions of the buffers. HABA was recrystalized from aqueous methanol.

C. Plant Material

The source of the plant material was the same as that detailed in section I. For analysis of seeds germinated for 1 and 2 days the embryos were excised from the endosperms, whereas whole shoots from 3 and 4 day old seedlings were excised at the point of seed attachment. To facilitate the isolation of embryos, seeds were dehusked essentially as described by Brock and Kannangara (1976). The seeds were soaked in 50% sulfuric acid at room temperature for 2 h with periodic stirring. They were then washed thoroughly with distilled water, during which time they were briskly stirred to loosen the adhering husks. The dehusked seeds were grown on filter paper in a clear plexiglass germination box. The box was placed in a growth chamber under a 12 cm water shield, with the growth chamber kept under continuous light at 600 ft-c and 20 C. The light intensity in the growth box was about 500 ft-c. Failure to use the water shield enabled the temperature within the germination box to reach 30 C. Under these conditions the mutant appeared temperature sensitive by remaining pale yellow. The seedlings did not recover when the box was opened at 4 days, and vermiculite added to cover the root systems and retain moisture, but bleached further becoming almost completely albino by 8 days. The normal was not affected under these conditions remaining dark green from 2 days onwards. The 12 cm water shield maintained the temperature near 20 C and the 4 day seedlings were visually comparable to the 4 day mutant seedlings grown in vermiculite in the same growth chamber. The seeds which were not dehusked were surface sterilized with sodium hypochlorite and grown in vermiculite as described in section I. The apical 3cm leaf segments were used for the analysis of 4',5' and 6' day old seedlings and for plastid isolation of 4, 6 and 8 day old seedlings.

D. Whole Tissue Analysis

Embryos or leaf segments were washed with distilled water and homogenized in 0.1 M potassium phosphate buffer (pH 8.3) containing 1 mM EDTA and 1 mM DTT, using a Ten Broeck tissue grinder. The homogenate was filtered through 1 layer of nylon cloth (Nitex) pore size 25 microns and centrifuged at 1,000g for 2 min. The supernatant was made to volume in the grinding buffer and aliquots taken for enzyme activity estimations and protein determinations.

E. Plastid Isolation

The isolation procedures used were essentially those described by Leese and Leech (1976). Leaf segments were washed with distilled water and chilled at 4 C for 15 min. All subsequent operations were carried out at 4 C. The leaf segments were homogenized in a 50 ml volume Waring blender with two successive 1 second bursts followed by one 3 second burst. The grinding medium was 67 mM phosphate buffer (KH_2PO_4/Na_2HPO_4) pH 8.0 containing 0.5 M sucrose, 1 mM $MgCl_2$ and 0.1% w/v BSA. For some experiments 30 mM Tricine-NaOH pH

8.0 was used in place of the phosphate buffer. To obtain adequate sample 3 to 4 grindings were required. The proportions used were approximately 4 ml grinding medium to 1 g fresh weight. The homogenate was gently squeezed through 2 layers of Miracloth (Calbiochem) and gravity filtered through 8 layers of 25 um pore size nylon cloth (Nitex). The filtrate was centrifuged for 2 to 5 min at 3,000g, 2 min for normal, 5 min for 4 day mutant, and 3.5 min for 6 and 8 day mutant. These schedules were found to give reasonable yields for the particular samples. The pellet was resuspended in a small volume of grinding medium and centrifuged through a 10 ml layer of 67 mM phosphate buffer, pH 8.0 containing 1 mM $MgCl_2$ and 0.6 M sucrose at 440g for 15 min in an IEC model BD-2 centrifuge using the 969 swing-out rotor. The chloroplast pellet was resuspended in 0.6 M buffered sucrose for plastid counts or disrupting buffer for the isolation of chloroplast fractions.

F. Plastid Counts

Plastids were counted with a haemocytometer viewed under a light microscope.

G. Bacterial Counts

Plastid isolates were monitored for bacterial contamination by plating serial dilutions on several agar types. These checks were performed by Dr. J.R. Williams, Plant Industry Laboratory, Alberta Agriculture.

H. Chloroplast Fractionation

The surface of the chloroplast pellet was rinsed gently with disrupting buffer and then suspended in the disrupting buffer, 0.02 M potassium phosphate (pH 7.0) containing 1 mM EDTA and 1 mM DTT, to give a chlorophyll concentration of 0.5 to 1.5 mg per ml. The suspension was transferred to a Ten Broeck tissue grinder and the plastids disrupted with 10 passes of the plunger. Stroma and lamellae fractions were separated by centrifugation for 1 h at 122,000g at 4 C in a Beckman Model E ultra-centrifuge using the number SW50E rotor. The pigment free stroma fraction was concentrated to approximately 1.3 mg protein per ml for enzyme activity studies, using an Amicon B15 miniconcentrator. The unwashed pellet was taken as the lamellar fraction, it was resuspended in the disrupting buffer to give a protein concentration of approximately 1.3 mg per ml.

I. Partial Purification of Acetyl CoA Carboxylase Subunits from *Escherichia coli*

E. coli B cells were cultured from single colonies on Bacto-Penassay Broth (Difco) and harvested at 1/2 to 3/4 log phase. The cells were supplied and grown by Dr. F.D. Cook, Soil Microbiology, University of Alberta. The cells were harvested by pelleting at 9,000g and washed once with 0.1 M potassium phosphate buffer, pH 7.0 containing 250 mM NaCl and collected again by centrifugation. They were then resuspended in a minimum volume of the phosphate buffered saline solution and the slurry transferred to screw-capped vials and frozen in dry-ice acetone mixture and stored at -20 C.

Acetyl CoA carboxylase subunits were partially purified by scaling down the method of Alberts _et al_. (1971) and Alberts and Vagelos (1968). All steps were performed at 4 C. The thawed cells were washed again with phosphate buffered saline solution and then suspended in 0.02 M potassium phosphate buffer, pH 7.0 containing 1 mM EDTA and 1 mM DTT; 0.5 g wet weight cells per ml of buffer (Hughes _et al_., 1971). Approximately 1 to 2 g of packed cells were suspended per test tube. The tubes were packed in an ice slurry and sonicated for 90 s at 30% maximum with a Sonic 300 Dismembrator (Artek Systems Corp., Farmingdale, New York, U.S.A.) using a pre-cooled microtip. Cellular debris was removed by centrifugation at 20,000g for 30 min (Figure 31).

The pooled supernatant fractions were made to volume and the nucleic acids were precipitated by adding a measured volume of 1 M $MnCl_2$ dropwise over 10 min with stirring to a final concentration of 0.05 M (Nozaki and Hayaishi,1971). The pH was maintained at 7 by the addition of 0.1 M KOH and the precipitate removed by centrifugation at 20,000g for 15 min. Solid ammonium sulfate was added to the supernatant, slowly with stirring over 30 min, to 45% saturation. The pH of the solution was again maintained at 7 with 0.1 M KOH. The solution was stirred an additional 15 min and the pellet collected by centrifugation at 20,000g for 30 min. The pellet was resuspended in 0.05 M imidazole-HCl (pH 6.7) to a protein concentration of approximately 20 mg per ml. Alumina Cγ, which was preequilibrated in the imidazole buffer, was added at a gel to protein ratio of 1/25. The protein-gel mixture was stirred for 5 min and the gel collected by centrifugation at 12,000g for 3 min. An additional volume of gel equal to the first volume was then added to the supernatant and treated as above. The supernatant fraction was saved for isolation of the carboxyltransferase constituent. The two gel fractions were pooled and washed with 3 successive 200 ul portions of 0.5 M ammonium sulfate in 0.1 M Tris-HCl, pH 7.7, per mg of gel. For each wash the gel was stirred for 10 min and collected by centrifugation. The biotin carboxylase and biotin carboxyl carrier protein subunits (Ea) were eluted from the alumina with 0.4 M potassium phosphate, pH 7.7 (Alberts and Valgelos, 1968). Three successive washes with 400 ul of buffer per mg gel treated as previously described were used to elute the Ea constituent. The supernatants were pooled and concentrated by precipitation with solid ammonium sulfate to 50% saturation.

Using the conditions described the carboxyltransferase constituent of the enzyme complex (Eb) is only lightly absorbed if at all to the gel and most of this is eluted with the 0.5 M ammonium sulfate solution (Alberts _et al._, 1971). The combined ammonium sulfate washes and the initial alumina Cγ supernatant were applied to an hydroxylapatite column (10 ml column volume / 10 mg protein). The hydroxylapatite was previously equilibrated with 0.01 M potassium phosphate, pH 7.7. The column was eluted in sequence with 0.01 M potassium phosphate, pH 7.7 and 0.2 M potassium phosphate pH 7.7 until the OD at 280 nm returned to the base line, approximately 2 column volumes each fraction (Figure 32). The fraction containing the carboxyltransferase component was then eluted with 0.4 M potassium phosphate, pH 7.7 (Figure 32) and concentrated by precipitation with the addition of solid ammonium sulfate to 50% saturation.

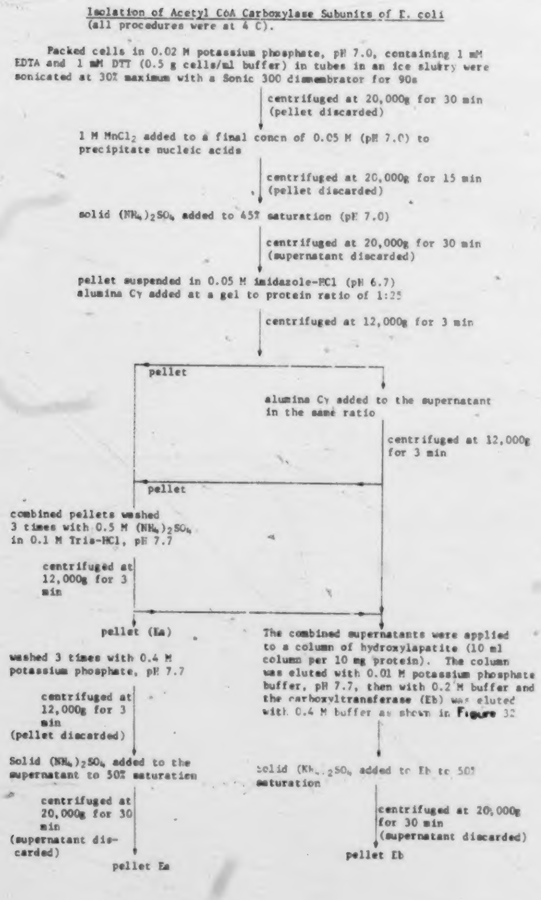
J. Assay System for Acetyl CoA Carboxylase Activity

Enzyme activity was assayed by the acetyl CoA dependent incorporation of (^{14}C)-bicarbonate into an acid stable product. All constituents, excluding the enzyme preparations, were made up as individual solutions in deionized water and stored at -20 C until required. Solutions of ATP and $MgCl_2$ were brought to pH 8.2 (20C) with NaOH. Samples of $NaH^{14}CO_3$, $NaHCO_3$ and acetyl CoA sufficient for a single experiment were stored in glass sealed vials and screw capped vials respectively. They were thawed just before use. Solutions of ATP, DTT, and BSA were refrozen a maximum of 3 to 4 times. The reaction mixture contained in a final volume of 200 ul: 5 umol Tris-HCl, pH 8.3 ; 1 umol ATP; 2 umol $MgCl_2$; 40 mmol KCl; 0.50 umol DTT; 0.2 mg BSA;

Figure 31. Brief outline of procedure used for partial purification of acetyl coenzyme A carboxylase subunits from *E. coli* cells.

Figure 32. Partial purification of the carboxyltransferase subunit of acetyl coenzyme A carboxylase from *E. coli* cells. Whole cell homogenate, prepared as outlined in methods and in Figure 31, was applied to an hydroxylapatite column and eluted in sequence with 0.01 M potassium phosphate buffer pH 7.7 and 0.2 M potassium phosphate buffer pH 7.7. The carboxyltransferase enriched fraction was then eluted with 0.4 M potassium phosphate buffer, pH 7.7 (fraction C).

Figure 31



Isolation of Acetyl CoA Carboxylase Subunits of E. coli
(all procedures were at 4 C).

Packed cells in 0.02 M potassium phosphate, pH 7.0, containing 1 mM EDTA and 1 mM DTT (0.5 g cells/ml buffer) in tubes in an ice slurry were sonicated at 30% maximum with a Sonic 300 dismembrator for 90s

centrifuged at 20,000g for 30 min (pellet discarded)

1 M $MnCl_2$ added to a final concn of 0.05 M (pH 7.0) to precipitate nucleic acids

centrifuged at 20,000g for 15 min (pellet discarded)

solid $(NH_4)_2SO_4$ added to 45% saturation (pH 7.0)

centrifuged at 20,000g for 30 min (supernatant discarded)

pellet suspended in 0.05 M imidazole-HCl (pH 6.7)
alumina Cγ added at a gel to protein ratio of 1:25

centrifuged at 12,000g for 3 min

pellet

alumina Cγ added to the supernatant in the same ratio

centrifuged at 12,000g for 3 min

pellet

combined pellets washed 3 times with 0.5 M $(NH_4)_2SO_4$ in 0.1 M Tris-HCl, pH 7.7

centrifuged at 12,000g for 3 min

pellet (Ea)

washed 3 times with 0.4 M potassium phosphate, pH 7.7

centrifuged at 12,000g for 3 min (pellet discarded)

Solid $(NH_4)_2SO_4$ added to the supernatant to 50% saturation

centrifuged at 20,000g for 30 min (supernatant discarded)

pellet Ea

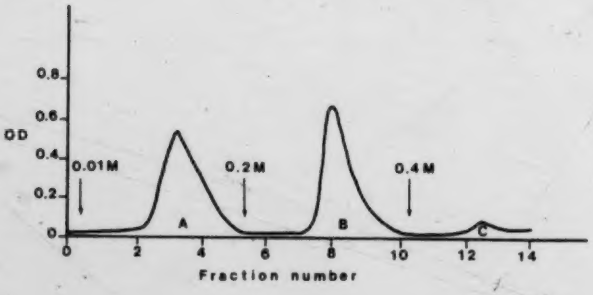
The combined supernatants were applied to a column of hydroxylapatite (10 ml column per 10 mg protein). The column was eluted with 0.01 M potassium phosphate buffer, pH 7.7, then with 0.2 M buffer and the carboxyltransferase (Eb) was eluted with 0.4 M buffer as shown in Figure 32

Solid $(NH_4)_2SO_4$ added to Eb to 50% saturation

centrifuged at 20,000g for 30 min (supernatant discarded)

pellet Eb

Figure 32

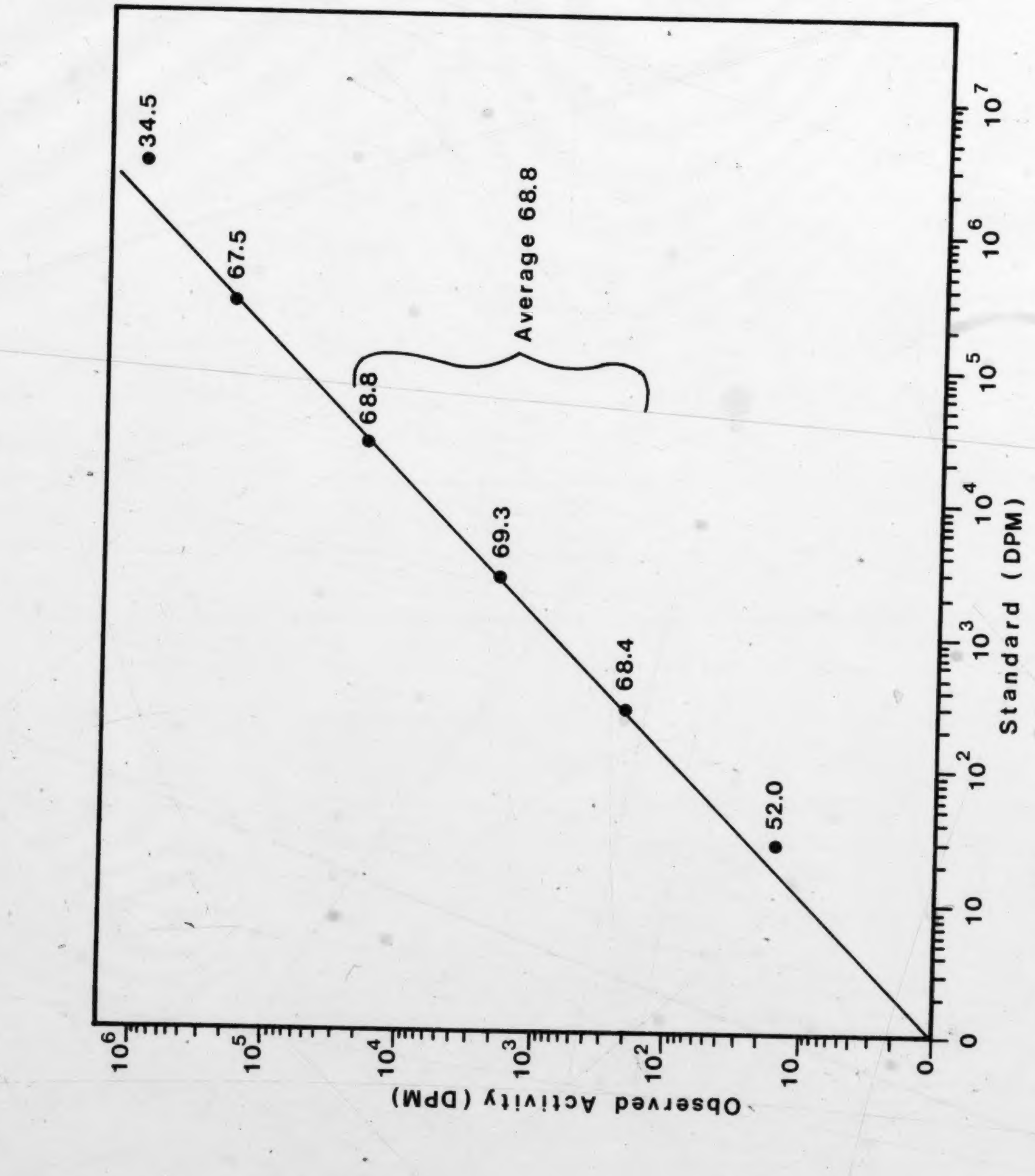


0.74 umol $H^{14}CO_3$ (5 uCi); 0.5 umol acetyl CoA and plant extract containing 10 to 40 ug of protein. In all cases 2 to 3 replications at differing protein concentrations were employed and the reaction rates were proportional to the protein concentrations over the range used. Acetyl CoA was added to initiate the reaction after the other constituents had equilibrated for 3 min at 30 C. The incubations were performed in a fume hood on a shaking water bath and the reactions terminated after 10 min by the addition of 40 ul of concentrated HCl. Fifty ul aliquots of the assay mixture were spotted on Whatmann #1 filter paper discs, 21 mm diameter, and dried in a fume hood with the aid of a heat lamp at 60 C for 30 min. The acid stable radioactivity was counted in a scintillation counter using 0.4% PPO and 0.01% POPOP in scintillation grade toluene as the scintillation fluid. Samples lacking acetyl CoA served as controls.

The specific activity, in DPM per umole, of the $NaH^{14}CO_3$ solution was determined as outlined by Miller and Levy (1969). Serial dilutions of the labeled solution from $2.75X10^6$ to 2.75X10 DPM were made with 0.1 M NaOH and aliquots were spotted on filter paper, dried and counted in the same manner as the reaction mixtures. The standard curve obtained is shown in Figure 33. In the dilution range $2.75X10^5$ to $2.75X10^2$ DPM the counting efficiency was 68.8% and this value was used in calculating the specific activities..

Protein estimations were determined by the method of

Figure 33. Standard curve used to determine specific activity of 1-^{14}C sodium bicarbonate solution. Dilutions of 2.75 x 10^6 to 2.75 x 10 DPM in 0.1 M NaOH were prepared and counted as outlined in methods.


Observed Activity (DPM)
0
10
10^2
10^3
10^4
10^5
10^6
Standard (DPM)
0
10
10^2
10^3
10^4
10^5
10^6
10^7
52.0
68.4
69.3
68.8
67.5
34.5
Average 68.8

Bradford (1976) with BSA fraction V as the standard and the chlorophylls were quantified according to Arnon (1949).

K. Identification of the Reaction Product

The reaction product was analyzed by Gelman TLC as described by Huang(1970). The reaction was terminated with concentrated HCl and then brought to 3 N KOH to hydrolyze the thioester, or terminated with 6 N KOH. Hydrolysis was allowed to proceed for 6 to 8 h and the samples neutralized to pH 7 with HCl. Aliquots of unhydrolyzed, hydrolyzed and malonic acid standard were spotted on Gelman ITLC-SG sheets, dried in a fume hood at 60 C for 15 min under a heat lamp and developed with the solvent system of water-saturated ether:formic acid (7:1, v/v). After chromatography the sheets were dried in the fume hood at 60 C for 30 min.

Unlabeled malonic acid was detected with slightly alkaline bromocresol green solution, 400 mg/liter in 95% ethanol (Denison and Phares, 1952). For radioactivity determinations, 1 cm sections were cut from the sheet and the activity determined in a scintillation counter as previously described.

L. Biotin Determination

The biotin content of the chloroplast stroma and lamellar fractions were determined by a spectrophotometric assay based on the binding of a dye by avidin, essentially as outlined by Green (1970). Stroma fractions were defatted with 2 washings of diethyl ether, lamellar fractions were resuspended in 1-2 ml of 50 mM potassium phosphate buffer, pH 6.8 using the Ten Broeck tissue grinder and defatted with diethyl ether:methanol (4:1, v/v). The stroma and lamellar fractions were then made to a volume of 5 or 10 ml with 50 mM potassium phosphate buffer, pH 6.8 and heat denatured at 70 C for 15 min. After cooling to room temperature, the samples were incubated with 10 mg protease for 48 h at 30 C. The samples were again heat denatured and then centrifuged at 12,000g for 30 min. The supernatant was freeze dried and the biotin content determined by a modification of the Green method (Gerwin _et al_, 1969).

The freeze dried samples were dissolved in 450 ul of 0.1 M phosphate buffer pH 6.8 and 150 ul containing 242 ug avidin and 22 ug HABA was added. The absorbance was read at 500 nm and from this the absorbance at 600 nm was subtracted. Biotin was quantified by comparison to standards which were also subjected to the protease digestion. Representative absorption scans and standard curves of biotin standards with and without the protease digestion are shown in Figures 34 and 35 respectively.

Protein estimates, by the method of Lowry _et al_. (1951), following precipitation with 5% TCA, on aliquots taken before and after the 48h protease digestion indicated that 85% of the total proteins were digested.

Figure 34. Absorption scans and standard curve of biotin standards quantified with the avidin dye binding method. The blank and biotin standards contained 242 µg avidin and 22 µg 2(4-hydroxazobenene) benzoic acid (HABA) in a final volume of 600 µl. HABA lacked avidin and biotin. The readings for the standard curve (B) were taken at 500 nm.

Figure 35. Absorption scans and standard curve of biotin standards following protease digestion. Same as Figure 34 only biotin standards were incubated with protease as outlined in methods.

Figure 34

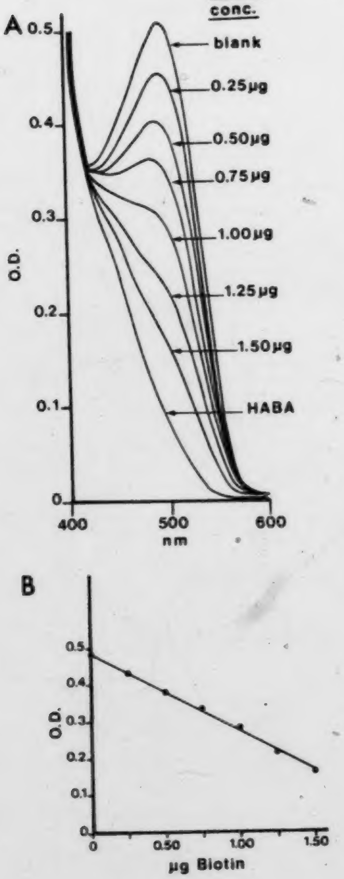
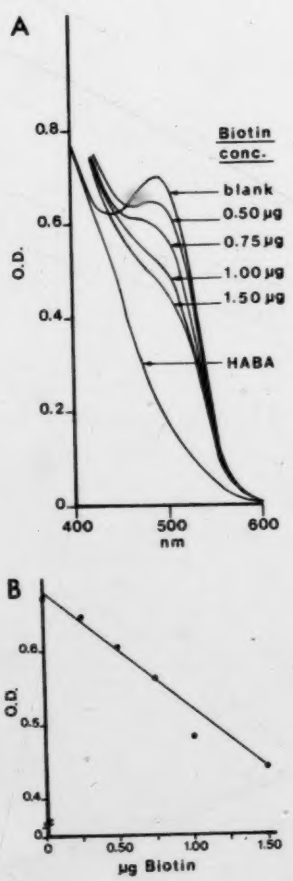


Figure 35



VII. RESULTS

A. Acetyl CoA Carboxylase Activity of Whole Tissue Homogenates

The acetyl CoA carboxylase activity of whole cell homogenates is shown in Table VII. The mutant and normal showed similar activities during the initial 3 days of growth when compared on the basis of protein content, fresh weight and seedling count. A marked increase in specific activity from approximately 6 to 20 nmoles/mg protein/min and on a per 100 seedling basis from approximately 12 to 43 umoles/100 seedlings/h occurred during this interval. Increases were, however less evident when expressed on a fresh weight basis. By 4 days the activity of the mutant exceeded that of the normal on all comparisons. These differences were more evident on samples taken from the apical 3 cm leaf segments at 4', 5' and 6' days. The difference between the 4 and 4' days, which is most evident for activity expressed as seedling counts, is likely due mainly to the sampling method as outlined in materials and methods. On samples taken from leaf segments (4', 5' and 6' days) the activity of the normal was maximal at 4' days and showed a rapid decline at 5' and 6' days. The activity of the mutant peaked at 5' days and showed a decline by 6' days. The activity of the mutant at 6' days was only slightly lower than that of the 4' day normal. The 6 day mutant contained approximately twice the activity of the 6 day normal on both protein and plant count comparisons.

B. Soluble Leaf Acetyl CoA Carboxylase

The acetyl CoA carboxylase enzyme in higher plants has been reported as a completely soluble protein in wheat germ extracts (Hatch and Stumpf, 1961; Heinstein and Stumpf, 1969) and in extracts from barley embryos (Brock and Kannangara, 1976). Kannangara and Stumpf (1972), (1973) and Kannangara and Jensen (1975) suggest that the enzyme in plant chloroplasts is the prokaryote type in that it is dissociable into three constituents; biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC) and carboxyltransferase (CT) similar to that of E. coli (Vagelos, 1974). The BC and CT were suggested to be stromal enzymes, while the BCCP moiety was membrane bound. An increase in the specific activity of acetyl CoA carboxylase in the high speed supernatant fraction was found in this study following centrifugation of 5 day normal Gateway barley (apical 5cm) leaf homogenates at 110,000g for 60 min. The results in nmoles $NaHCO_3$ incorporated per mg protein were:

Whole Leaf Homogenate......	9.6
110,000g Supernatant......	17.1
110,000g Pellet............	1.4

This is contrary to what would be expected if one of the

Table VII. Acetyl CoA carboxylase activity of whole cell homogenates of mutant and normal barley seedlings from 1 to 6 days of age expressed on protein, fresh weight and per seedling basis. Days 1 to 4 are for seedlings germinated from dehusked barley seeds and harvested as outlined in materials and methods. Days 4´ to 6´ are for the apical 3 cm leaf segments of seedlings grown on vermiculite.

The complete reaction mixture contained in a final volume of 200 µl : 5 µmol Tris-HCl pH 8.3; 1 µmol ATP; 2 µmol $MgCl_2$; 40 mmol KCl; 0.50 µmol DTT; 0.2 mg BSA; 0.74 µmol $NaH^{14}CO_3$ (5 µCi); 0.5 µmol acetyl CoA and plant extract containing 10 to 40 µg of protein. Samples lacking acetyl CoA served as controls. Details of the procedures are as outlined in materials and methods.

Values for 1 to 4 day plant material are the mean ± SE of two experiments with 3 replicates per experiment. Values for 4´ to 6´ days are the mean ± SE of one experiment with 2 or 3 replications.

Acetyl CoA Carboxylase Activity

HCO_3 Incorporated Mutant	Days 1	2	3	4	4′	5′	6′
n mol/mg protein/min	6.50 ± 0.21	7.46 ± 0.72	20.38 ± 1.54	25.53 ± 1.21	19.63 ± 0.23	19.93 ± 1.48	12.53 ± 0.79
μ mol/gm fw/h	23.48 ± 1.97	16.05 ± 0.68	28.28 ± 3.14	24.58 ± 0.78	23.17 ± 1.37	21.37 ± 0.96	16.57 ± 0.92
μ mol/100 seedlings/h	12.50 ± 0.94	25.92 ± 3.56	40.72 ± 2.92	84.90 ± 7.68	42.70 ± 2.55	44.33 ± 2.33	33.97 ± 2.38
Normal							
n mol/mg protein/min	5.82 ± 0.15	8.03 ± 0.46	19.28 ± 0.80	20.84 ± 1.18	15.25 ± 1.65	10.87 ± 1.22	6.40 ± 0.49
μ mol/gm fw/h	24.16 ± 2.16	16.32 ± 1.31	25.67 ± 1.16	20.30 ± 0.81	21.33 ± 1.98	15.90 ± 1.57	10.03 ± 0.92
μ mol/100 seedlings/h	15.52 ± 1.05	33.72 ± 3.09	43.06 ± 4.34	75.85 ± 6.60	37.80 ± 3.52	29.13 ± 2.90	16.97 ± 1.58

constituents of the enzyme complex were membrane bound and is in agreement with the results of Reitzel and Nielsen (1976) who reported an increase in the specific activity when whole barley leaf homogenates were centrifuged at 150,000g for 45 min.

C. Chloroplast Acetyl CoA Carboxylase

Since several studies have indicated that the plastid fraction is the major site of fatty acid synthesis (Smirnov, 1961; Mudd and McManus, 1962; Stumpf and James, 1963; Weaire and Kekwick, 1975; Ohlrogge et al. 1979), chloroplasts were isolated and the enzyme activity was assayed in both membrane and stromal fractions. These results shown in Table VIII suggest that the complete enzyme complex occurs in the soluble fraction of the chloroplast. Further comparisons between the mutant and normal were therefore made using isolated plastids.

Chlorophyll Content per Plastid

In an attempt to obtain a suitable parameter for comparisons, plastid numbers were determined for both the normal and mutant plant types. Since chlorophyll is confined to the chloroplast membranes, calibration curves were established expressing chlorophyll concentration against plastid number. A typical curve is shown in Figure 36. Such curves were established for both mutant and normal seedlings at 4, 6 and 8 days. From the slope of each line the number of plastids corresponding to the chlorophyll concentration

Table VIII. Acetyl CoA carboxylase activity of chloroplast stroma and membrane fractions isolated from the apical 3cm leaf segments of 4 and 5 day old normal barley seedlings. Complete reaction mixture given in Table VII.

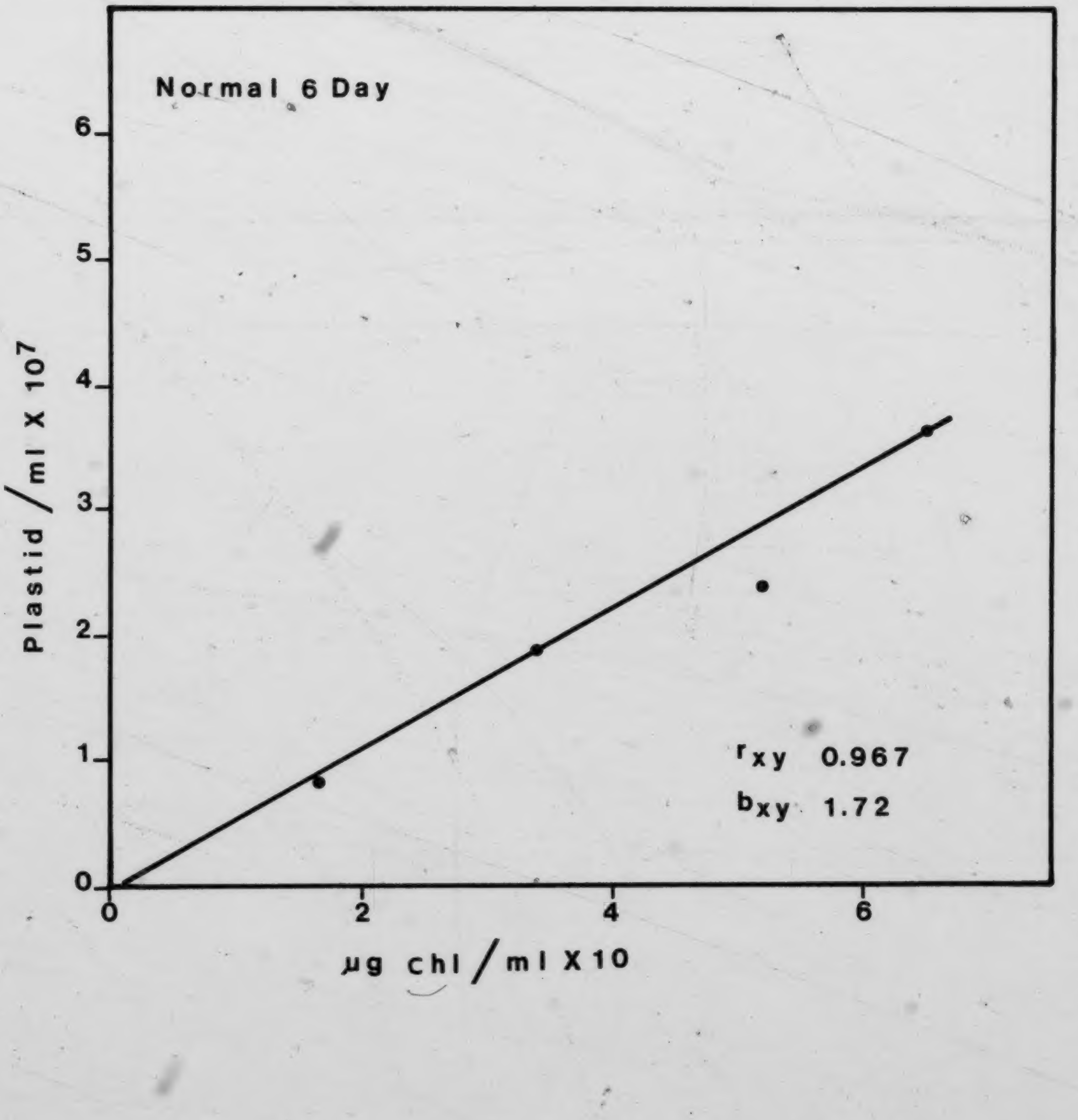
Chloroplast Fraction	nmoles HCO_3 incorporated/ mg protein/min Growth Stage (days) 4	 5
Whole(disrupted)	4.33± 0.98	6.45
Stroma	24.30± 4.22	19.02
Lamellar	1.28± 0.27	0.62
Stroma + Lamellar	6.00± 1.37	7.84

Values for 4 days are the mean± SE of 3 determinations.
Values for 5 days are single estimates.

could then be determined. The chlorophyll content per plastid for both plant types at 4, 6, and 8 days is shown in Table IX.

The chlorophyll content per plastid of the normal reached a maximum at 6 days and showed a slight decline by 8 days. The plastid chlorophyll content of the mutant was still increasing at 8 days, where it represented 54% of the level reached by the normal at its maximum. A comparison of the ratio of chlorophyll content of the mutant to the normal on a whole leaf and on a per plastid basis (Table IX) suggests some selection occurred during plastid isolation. This is especially evident at 4 days where on the fresh weight comparison of whole leaves the chlorophyll content of the mutant was approximately 8% of the level reached by the

Figure 36. Regression line used to determine the number of plastids from the chlorophyll concentration of an isolated plastid fraction of 6 day normal barley. At each growth stage three separate analyses were conducted for the normal and the mutant. The average of the three was used to obtain the individual regression lines.


Normal 6 Day
Plastid /ml X 10^7
0
1
2
3
4
5
6
0
2
4
6
μg chl /ml X10
r_{xy} 0.967
b_{xy} 1.72

normal, but represented 38% on the per plastid comparison. These differences were however less evident by 8 days presumably due to the general increase in the mutant plastid size and the overall mutant chloroplast population becoming more homogeneous.

Bacterial Contamination in Plastid Isolation

Bacterial contamination in the plastid isolates was minimal as shown in Table X. Using the highest counts obtained for the isolates monitored it was estimated at 0.007% and 0.04% for the 6 day normal and mutant respectively. Those of the 8 day were slightly lower representing 0.0008% for the normal and 0.002% for the mutant.

Enzyme Activity of Chloroplast Stroma Preparations

The enzyme activity obtained in chloroplast stromal preparations from both plant types at 4, 6 and 8 days is shown in Table XI. The activity of the mutant was very high at 4 days on all comparisons; protein content, chlorophyll concentration and plastid number. The activity in nmoles/mg protein/min declined to one third by 8 days, whereas on a chlorophyll basis it decreased to one ninetieth during this interval. The activity of the normal was also highest at 4 days and decreased to one sixth on a protein basis and one hundredth on a chlorophyll basis by 8 days. Comparisons between the two plant types at the interval of maximum activity, 4 days, showed the normal contained only 60% of the activity present in the mutant when expressed as

Table IX. Chlorophyll content per plastid, from the apical 3cm leaf segments of normal and mutant barley seedlings at 4, 6 and 8 days, and the percentage chlorophyll in the mutant relative to the normal expressed on a fresh weight and a per plastid basis.

Days	pgChl/Plastid		% ChlM/chlN	
	Normal	**Mutant**	**Whole Leaf**	**Plastids**
4	1.35 ±0.18	0.51 ±0.05	8.16	37.8 ±1.9
6	1.74 ±0.04	0.70 ±0.11	26.2	40.2 ±7.3
8	1.56 ±0.05	0.94 ±0.10	48.6	60.2 ±6.8

Results are the mean ±SE for 3 experiments with 3 to 4 concentrations used per experiment as shown in Figure 36. Whole leaf estimates are averages of two experiments.

Table X. Estimation of numbers of bacteria and plastids from chloroplast fractions prepared from 6 and 8 day mutant and normal barley seedlings. Bacteria counts were estimated from colonies formed after plating serial dilutions of plastid preparations on 4 different agar media and plastid counts from the same preparation were determined using a haemocytometer.

	Bacteria/ml $x10^4$				**Plastids/ml** $x10^8$
Agar	King's	Burkhold's	Potato Sucrose	Nutrient	
Mutant (days)					
6	8.0	5.0	4.5		2.0
8				6.1	3.5
Normal (days)					
6	2.5	0.7	1.4		3.4
8				0.6	4.5

Bacteria counts represent the highest values obtained, using 3 dilutions with 3 plates per dilution.
Plastid counts are the averages of 3 estimates.

Table XI. Acetyl CoA carboxylase activity of chloroplast stromal preparations from apical 3 cm leaf segments of 4, 6 and 8 day old normal and mutant barley seedlings. The activity is expressed on a protein, chlorophyll and per plastid basis. Also the soluble protein to chlorophyll ratios of the isolated plastids and an estimate of the chlorophyll content of the leaf segments was determined. Complete reaction mixture is given in Table VII.

Enzyme activity values are the mean ±SE of 2 experiments with 2 to 3 replicates per experiment.

Enzyme Activity of Chloroplast Stroma Preparations

Days	n moles HCO_3 Incorporated			Protein	mg chl
Mutant	/mg protein/min	/mg chl/min	/plastid x10^8/min	/chl	/g fw
4	54.2 ± 1.8	1730 ± 178	88.3 ± 9.1	32.6 ± 5.2	0.12 ± 0.02
6	34.3 ± 0.8	163 ± 38	28.4 ± 6.7	4.77 ± 1.9	0.62 ± 0.07
8	16.6 ± 1.3	19.0 ± 2.8	1.79 ± 0.3	1.18 ± 0.2	1.08 ± 0.03
Normal					
4	31.4 ± 3.1	120.7 ± 17	16.3 ± 2.3	4.0 ± 0.7	0.99 ± 0.14
6	7.71 ± 0.5	4.08 ± 0.5	0.51 ± 0.2	0.90 ± 0.3	1.98 ± 0.27
8	4.76 ± 0.3	1.13 ± 0.1	0.18 ± 0.02	0.25 ± 0.05	2.10 ± 0.03

nmoles/mg protein/min and only 7% when expressed as nmoles/mg chlorophyll/min. Since the chlorophyll and protein content change considerably during this growth interval results were also expressed in terms of plastid number. The chlorophyll content on a leaf fresh weight basis shows a 9 fold increase in the mutant and a 2 fold increase in the normal during this growth interval (Table XI, Figure 13). The protein content also increased (Figure 12) and ribulose 1,5-bisphosphate carboxylase, the major chloroplast stromal protein, increased 1.5 and 5 fold respectively for the normal and mutant between 4 and 8 days (Barankiewicz _et al._, 1979).

When activity was expressed on plastid number (Table XI) both plant types again showed maximum activity at 4 days, however the normal contained only 18% of the level reached by the mutant. The activity in both decreased to very low levels by 8 days. This decrease could likely be accounted for in part by the loss of chloroplast soluble proteins during plastid isolation and also perhaps due to an inhibitor build-up during greening (Burton and Stumpf, 1966; Kannangara and Stumpf, 1972). Estimates of soluble protein/chlorophyll ratios of the plastid isolates (Table XI) were considerably lower for the mutant at 8 days and the normal at 6 and 8 days than the protein/chlorophyll ratios reported for intact isolated plastids; 6.2 for spinach (Kirk, 1971); 5.2 to 6.4 for tobacco (Ongun _et al_, 1968); 8.9 for spinach (Joyard and Douce, 1977). Also a cursory comparison of the ability to evolve O_2 in the presence of ferricyanide before and after osmotic shock (Lilly *et al.*, 1975) of a 7 day normal plastid preparation suggested only 25% of the plastids were intact.

Tests to Determine the Presence of an Inhibitor

Kannangara and Stumpf (1972) reported the carboxyltransferase reaction was inhibited in disrupted spinach chloroplast preparations and substantial formation of malonyl CoA occurred upon the addition of the carboxyltransferase subunit purified from *E. coli*. The addition of the partially purified *E. coli* carboxyltransferase subunit (Eb) to a 5 day normal stromal preparation did not enhance $NaH^{14}CO_3$ incorporation (Table XII) suggesting the inhibitor was not present, or if present it readily inactivated the carboxyltransferase subunit. In a further test for inhibitor activity aliquots from normal and mutant stromal preparations were pooled. Also whole tissue homogenates from 9 day normal leaves and 1 day normal embryos were pooled. Since the pooled activities as shown in Table XIII were additive the lower activity from the normal stromal preparations were not due to the action of an inhibitor. However comparisons between whole tissue homogenates from 9 day normal leaves and 1 day normal embryos revealed an inhibitory effect.

Table XII. Effect of the addition of E. coli carboxyltransferase subunit to a stromal preparation from 5 day normal barley on the acetyl CoA carboxylase activity. Activity was determined as the acetyl CoA dependent incorporation of $NaH^{14}CO_3$ into an acid stable product.

EnzymeSystem	Activity Incorporated (DPM)
Ea(E.coli)	3,011
Eb(E.coli)	45
Ea+Eb	77,109
stroma	30,669
stroma+Eb	29,700

The complete reaction mixture is given in Table VII. Eb=23ug protein; stroma preparation=75ug protein.

Table XIII. Effect of pooling stromal preparations and whole tissue homogenates of barley seedlings on acetyl CoA carboxylase activity. Stromal preparations from 4, 6 and 8 day old mutant and normal barley seedlings were pooled. Whole tissue homogenates of 9 day normal leaf and one day normal embryo were also pooled.

	nmoles/mg protein/min			
	Stroma Preparations (M+N)			Whole Tissue Homogenate[1] (N+N)
days/	4	6	8	9+1
Activity Obtained	47.4(2)	18.1(3)	13.2(2)	1.2(1)
Activity Expected[2]	41.6(2)	20.8(3)	11.3(2)	1.9(2)

Numbers in brackets indicate the number of determinations
The complete assay system is given in Table VII.

[1] assay system did not contain KCl, DTT, BSA.

[2] expected values are the mean of the specific activities of the unpooled enzyme preprations.

D. Assay Constituents

The effect of the various assay constituents on the enzyme activity is shown in Table XIV. The absolute requirement for ATP, $MgCl_2$ and acetyl CoA is in agreement with the known cofactor requirements for acetyl CoA carboxylase activity (Lane et al., 1974). The mild stimulation by KCl, DTT, and BSA was not always experienced and in some instances as shown in Table XIV, BSA at 0.2 mg was slightly inhibitory.

E. Inhibitory Effect of Avidin

Avidin completely inhibited enzyme activity as shown in Table XV. This inhibition was prevented if the reaction medium containing avidin was preincubated in the presence of biotin before the addition of $NaH^{14}CO_3$. This observation indicated biotin was participating in the reaction. Biotin is known to serve as a prosthetic group covalently bound to the enzyme (Lane et al., 1974; Volpe and Vagelos, 1976; Bloch and Vance, 1977).

F. End product Analysis

Further evidence that the incorporation of $NaH^{14}CO_3$ was a measure of acetyl CoA carboxylase activity is provided by end-product analysis. As shown in Figure 37, thin layer chromatography on ITLC-SG plates suggested the presence of a CoA ester since the activity remained at the origin in the solvent system used (Huang, 1970). Upon hydrolysis the

Table XIV. Cofactor requirements and the effect of other additives on acetyl CoA carboxylase activity. The complete assay system is given in Table VII.

Assay Mixture	Activity Incorporated (DPM)	
	6 day normal	2 day normal
Complete	6,093	4360
-ATP	57	185
-$MgCl_2$	20	49
-KCl (or 20mmol KCl)	5,280(5,747)	5037
-DTT (or 0.25umol DTT)	5,708(6,054)	4414
-BSA (or 0.1mg BSA)	5,597(6,204)	5902
-KCl,-DTT,-BSA	5,031	--
-Enzyme	31	49
-$NaH^{14}CO_3$	26	12
-Acetyl CoA	41	66

The enzyme preparations added were 28ug protein from 6 day normal chloroplast stroma and 34ug protein from 2 day normal whole tissue homogenate prepared as outlined in methods. The values in brackets for KCl, DTT and BSA represent one half the concentrations of those used in the complete assay mixture.

radioactive compound showed the same migration pattern as malonic acid, which is the known acetyl CoA dependent acid stable product of acetyl CoA carboxylase activity.

G. Time Curve

The incorporation of $NaH^{14}CO_3$ was shown to be linear in time for an incubation period of approximately 20 min for chloroplast stromal preparations and 10 min for whole tissue homogenates of 1 day embryo (Figure 38).

Figure 37. Fractionation of the products of the acetyl CoA carboxylase enzyme assay from a 6 day mutant stromal preparation. The acid stable product was chromatogramed on ITLC-SG plates as outlined by Huang (1970). Alkaline hydrolysis was used to convert malonyl CoA to malonic acid plus CoA. The mobility of labeled malonic acid produced was compared with that of malonic acid standard. Unlabelled malonic acid was outlined by spraying with slightly alkaline bromocresol green solution. One cm segments of the developed chromatogram were counted in a scintillation counter to determine radioactivity. Unhydrolyzed represents an aliquot taken after the reaction was terminated with conc. HCl. Hydrolyzed represent an aliquot from the acid terminated mixture made to 3 N with KOH. After alkaline hydrolysis it was neutralized as outlined in methods.

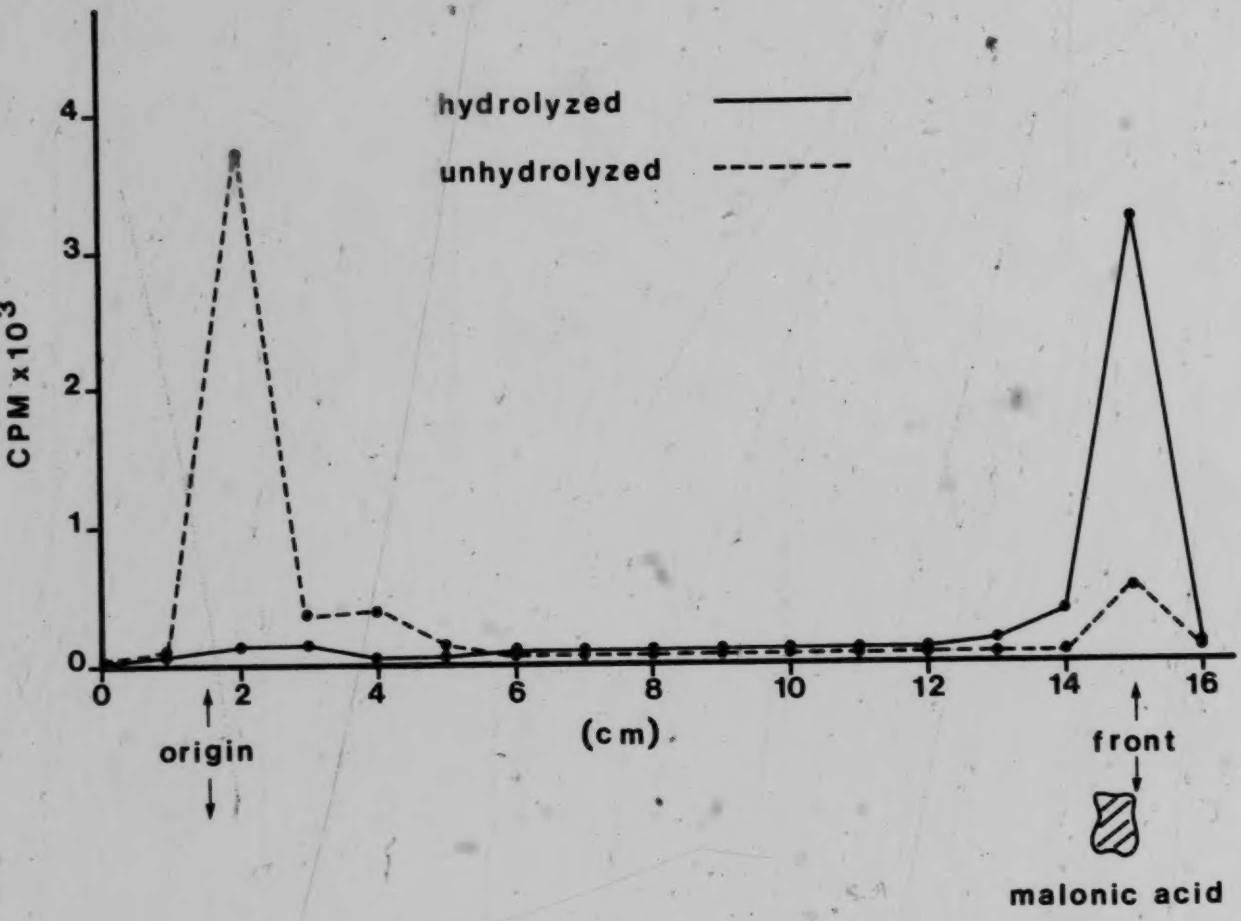
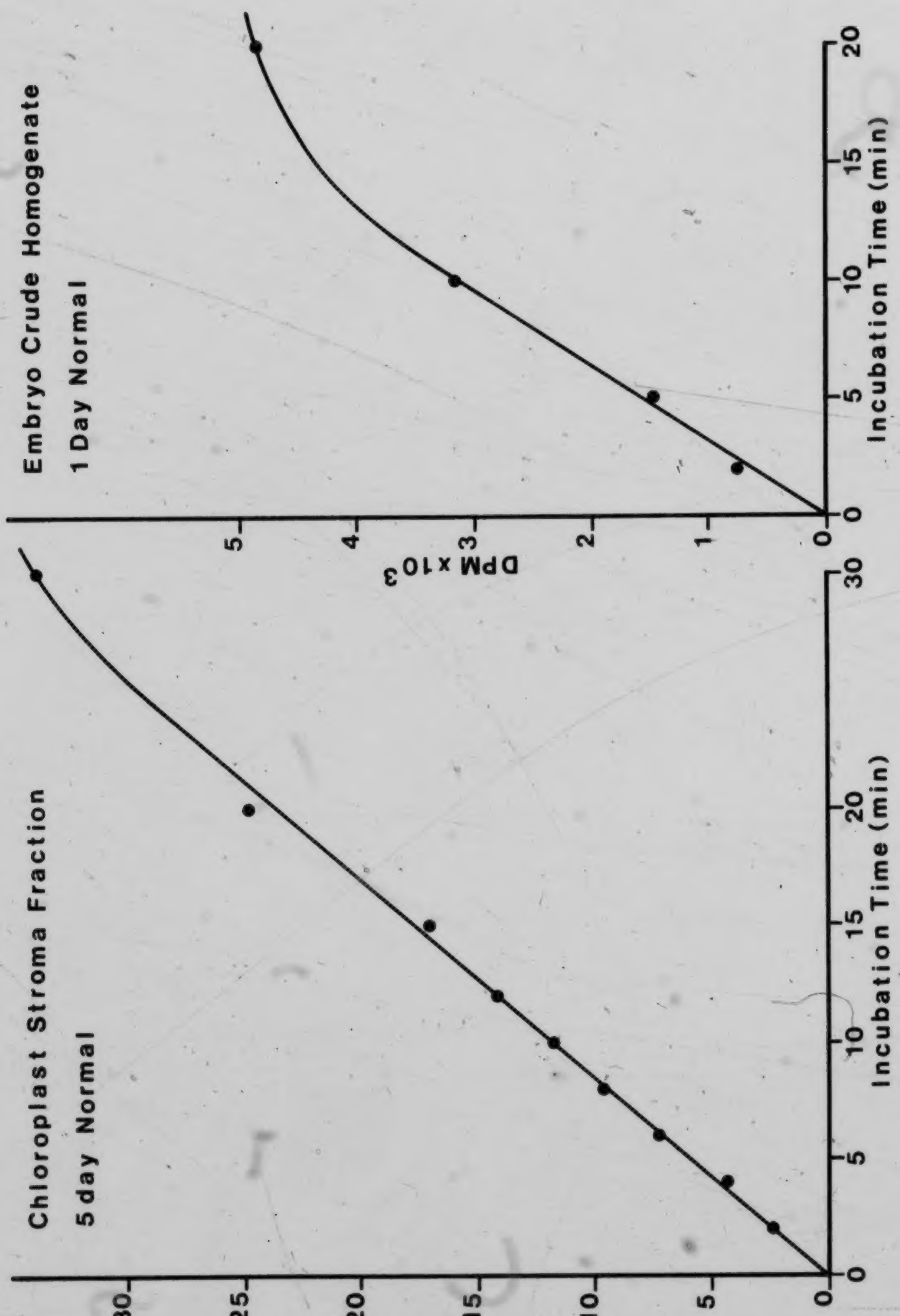

hydrolyzed
unhydrolyzed
CPM x10^3
0
1
2
3
4
0
2
4
6
8
10
12
14
16
(cm)
origin
front
malonic acid

Figure 38. Time curve for acetyl CoA carboxylase activity from normal barley. Chloroplast stromal fractions from 5 day normal barley leaf segments and whole cell homogenates from 1 day normal embryo were prepared as outlined in methods. Activities obtained for the stromal preparation between 2 and 20 min were: 20.0 ± 0.33 n moles/mg protein/min (mean ± SE, n=8) and for the embryo preparation between 2 and 10 min were: 5.75 ± 0.44 n moles/mg protein/min (mean ± SE, n=3).


Embryo Crude Homogenate
1 Day Normal
DPM x 10^3
0
1
2
3
4
5
Incubation Time (min)
0
5
10
15
20
Chloroplast Stroma Fraction
5 day Normal
DPM x 10^3
0
5
10
15
20
25
30
Incubation Time (min)
0
5
10
15
20
30

B. Biotin Content of Chloroplast Fractions

The biotin content in the stroma and lamellar preparations was also determined as a further test for the location of the enzyme within the chloroplast. As shown in Table XVI the biotin content was highest in the stromal fraction, although the unwashed membrane fraction also contained a considerable quantity. In contrast to the activity estimates the biotin content of the stromal preparations of the normal exceeded the concentration detected in the mutant.

Table XV. Effect of the addition of avidin[1] to stromal preparations of 5 day normal and 6 day mutant and to 2 day normal and mutant whole tissue homogenates on acetyl CoA carboxylase activity.

Assay medium[2]	Activity Incorporated (DPM)
5 day Normal (18ug protein)	
Complete	8,725
+Avidin (1.4units)	55
+Biotin (1.5ug) + Avidin (1.2units)[3]	7,589
6 day Mutant (38ug protein)	
Complete	19,989
+Avidin (1.1units)	43
+Biotin (1.6ug) + Avidin (1.1units)[3]	19,972
+Biotin (1.6ug)	19,088
2 day Normal (34ug protein)	
Complete	4,598
+Avidin (1.1units)	59
+Biotin (1.6ug) + Avidin (1.1units)[3]	3,433
2 day Mutant (29ug protein)	
complete	4,199
+Avidin (1.1units)	71
+Biotin (1.6ug) + Avidin (1.1units)[3]	3,495

[1] one unit binds 1 ug d-biotin

[2] complete assay medium as outlined in Table VII.

[3] the avidin and avidin-biotin mixtures were preincubated 3 min with the complete medium minus sodium bicarbonate $NaH^{14}CO_3$ was then added to initiate the reaction.

Table XVI. Biotin content of the chloroplast stromal and lamellar fractions from the apical 3cm leaf segments of 5 day mutant and normal barley seedlings.

Chloroplast Fraction	ug Biotin/mg Protein
Mutant	
Stroma	1.01±0.17
Lamellar	0.47±0.14
Normal	
Stroma	3.12±0.72
Lamellar	0.77±0.06

Assayed by the avidin-dye binding method (Green, 1970). Values represent the mean±SE of 3 experiments.

VIII. DISCUSSION

Acetyl CoA carboxylase catalyses the formation of malonyl CoA which is utilized by the fatty acid synthetase complex for *de novo* synthesis of fatty acids (Lane *et al.*, 1974; Stumpf, 1977). The carboxylase enzyme catalyses the first committed step in the synthesis of fatty acids and has been suggested to be the regulating enzyme in fatty acid synthesis (Lane *et al.*, 1974; Polakis *et al.*, 1973). In the present study the cofactor requirements (Table XIV), the complete inhibition by avidin (Table XV) and the end product obtained (Figure 37) corroborate the known reaction mechanism for acetyl CoA carboxylase.

The activities of the whole tissue homogenate of embryo isolates from one day seedlings of approximately 6 nmoles HCO_3 incorporated /mg protein/min (Table VII) are comparable to the activities reported for wheat germ (Heinstein and Stumpf, 1969) and barley embryo extracts (Brock and Kannangara, 1976) where the 30,000g crude supernatant fractions gave 6 and 3 nmoles/mg protein/min respectively. An increase in activity during growth as shown in Table VII was expected since acyl lipids are important constituents for membrane formation, however the nearly identical values of the mutant and normal at 2 and 3 days and the higher activity of the mutant at 4 days were not expected. Electron microscopic observations of 2 day shoot sections (data not presented) showed the normal shoots already contained developing chloroplasts with long lamellae traversing the plastid appressed in groups of up to 4 lamellae, whereas in the mutant shoots chloroplasts were not distinguishable. However, several small organelles containing starch grains were present which probably represent the undifferentiated proplastid (Kirk, 1967). The mutant at 4 days grown under very similar conditions was earlier shown to contain very little internal chloroplast membrane structure (Jhamb and Zalik, 1973). Reitzel and Nielsen (1976) in studies with barley mutants with leaky blocks in chlorophyll synthesis report the acetyl CoA carboxylase of the whole shoots was initially approximately 2/3 that of normal at 3 days and displayed a 2 day time lag before the onset of increased activity. Since the normal barley in this study was active at an earlier stage in chloroplast development and lipid synthesis in leaf segments is most active during the stage of chloroplast development (Hawke _et al._, 1974; Bolton and Harwood, 1978) an increased lipid synthesis above the level displayed for the mutant would be expected. Postulates for the initial high activity in the mutant could be increased mitochondria synthesis or alternative pathways for the enzyme product, malonyl CoA. The higher PE content as percentage of total acyl lipids of the mutant in comparison with the normal at 4 days (Figure 15) would suggest proportionally more mitochondrial to chloroplast membranes were present in the mutant since the mitochondria are enriched in PE and chloroplast membranes lack PE (Mazliak, 1977). However this proposed increased channeling of newly synthesized lipids into mitochondria is not evident when comparisons are made on a fresh weight basis since the PE content of both the mutant and normal are similar at 4 days (Figure 14).

Acetate labeling studies have indicated alternative pathways for acetate utilization in developing leaves of barley (Appelqvist _et al_., 1968), maize (Hawke _et al_., 1974) and isolated plastids from greening etiolated barley seedlings (Kannangara and Stumpf, 1971). During the initial stages in greening of barley leaf slices a substantial proportion of the ^{14}C-acetate label was directed into the steroid and nonsaponifiable lipid fraction in which β-amyrin was tentatively identified as the major labeled constituent (Appelqvist _et al_., 1968). The labeling pattern was reversed as chloroplast development progressed and 75% of the label occurred in the phospho-, sulfo- and galactolipids in the more developed tissue (Appelqvist _et al_., 1968). This study is of further interest since a constant level of acetate incorporation into the lipid fraction occurred during the greening studies (Appelqvist _et al_., 1968). Kannangara _et al_. 1971) reported that plastids isolated from barley leaves during the initial stages of illumination incorporated the majority of the chloroform/methanol extractable acetate label into the stromal fraction and 6-methyl salicyclic acid accounted for 50 to 70% of the ^{14}C-label. As greening progressed the flow of ^{14}C-label into 6-methyl salicyclic acid diminished sharply and most of the label was associated with the membrane bound lipids (Kannangara _et al._, 1971). The operation of alternative pathways was also evident in the study of Hawke _et al._ (1974) where appreciable synthesis of long chain saturated fatty acids with 20 or more carbon atoms were obtained in maize basal leaf segments which contained undifferentiated proplastids. Studies on the more mature leaf segments showed the C_{16} and C_{18} fatty acids were predominantly labeled and no detectable label occurred in the very long chain fatty acids. The long chain saturated fatty acids likely serve as precursors in the formation of cuticular wax (Kolattukudy, 1977). Steroid biosynthesis involves the isoprenoid pathway which does not require malonyl CoA (Appelqvist _et al._, 1968; Goodwin, 1977), thus a possible increased synthesis of steroids in the mutant relative to the normal could not account for the high acetyl CoA carboxylase activity of the mutant from days 2 to 4. Cuticular wax formation would likely be common to both mutant and normal. The formation of 6-methyl salicyclic acid, however was shown to require both CoA, CO_2 and ATP and the labeling pattern was consistent with the head to tail condensation of acetyl CoA with malonyl CoA (Kannangara _et al._, 1971a). The 6-methyl salicyclic acid was considered not a final end product in leaf tissue and is presumably accumulated as a result of the disruption of the normal sequence of "aromatic biosynthesis" (Kannangara _et al._, 1971a). Other cellular constituents which require malonyl CoA in their biosynthesis include biotin (Eisenberg, 1973) and flavonoids (Wong, 1976). The extent to which acetyl CoA carboxylase may take part in the flow of carbon into alternative pathways or a possible enrichment in the content of an acyl lipid class in the neutral lipid fraction of the mutant relative to the normal during initial leaf development was not further studied.

The lipid composition analysis (Figure 14) showed the accumulation of the major chloroplast lipids, the galactolipids MG and DG, increased rapidly up to 6 days in the normal but were still increasing rapidly at 8 days for the mutant. The acetyl CoA carboxylase activity expressed on seedling count (Table VII, days 4' to 6') partially reflects the lag in the mutant in which the highest activities in the apical 3 cm leaf segments occurred at 5' days whereas in the normal the highest for the 3 cm leaf segments was at 4' days and likely occurred earlier than 4' days (samples were not taken for the apical leaf segments younger than 4 days). The activities expressed on a seedling count basis (Table VII) are comparable to those of Reitzel and Nielsen (1975) in which the acetyl CoA carboxylase activity reached a maximum by 5 days of approximately 55 umoles/100 seedlings/h.

The acetyl CoA carboxylase activity of the chloroplast preparations (Tables VIII and XI) represents to the author's knowledge the first reported data showing appreciable acetyl CoA carboxylase activity from chloroplast isolates. Acetate labeling studies (Brooks and Stumpf, 1966) and more recently $^{14}CO_2$ labeling studies (Murphy and Leech, 1977; 1978) with isolated plastids indicated the label was readily incorporated into fatty acids suggesting the acetyl CoA carboxylase enzyme was functional. However, upon disruption of the chloroplasts acetate and acetyl CoA were ineffective substrates although malonyl CoA was readily incorporated (Brooks and Stumpf, 1966). Further studies indicated an inhibitor was present in the disrupted plastid fraction (Burton and Stumpf, 1966). However chloroplasts from immature spinach leaves isolated in a buffer system containing 0.06 M bicarbonate retained the ability to incorporate acetate into fatty acids and an acetyl CoA carboxylase activity of 1.7 nmoles/min/mg protein was reported (Kannanagara _et al._, 1973). Acetyl CoA carboxylase activity has also been reported in the proplastid of developing castor bean endosperm where the enzyme was shown to be a cytochemical marker for the proplastid fraction. A specific activity as high as 10.7 nmoles/min/mg protein was reported for this fraction (Burden and Canvin, 1975).

The very high activity of the 4 day mutant stromal preparation relative to the normal, based both on chlorophyll and protein content is likely mainly due to the much higher RUBP-carboxylase protein present in the normal relative to the mutant at 4 days (Barankiewicz _et al._, 1979) and the very low chlorophyll content per plastid of the 4 day mutant (Table IX). Accurate comparisons on a per plastid basis are not possible due to the large differences in the protein/chlorophyll ratios of the isolated plastids (Table

XI). The very high protein/chlorophyll ratio of the 4 day mutant would reflect its low chlorophyll content per plastid. The decline in the protein/chlorophyll ratio which is most evident in the 6 and 8 day normal and 8 day mutant likely indicates excessive loss of soluble protein from the more mature plastids during isolation. The plastid isolation procedure was similar to that outlined by Leese and Leech (1976), who reported high recovery of intact chloroplasts from maize. However, unlike their procedure the final centrifugation buffer did not contain BSA in order to permit enzyme activity comparisons on a protein basis. The isolation of intact chloroplasts from barley and other grasses is difficult due to their high fiber content. Recently the use of a kitchen homogenizer modified to contain razor blades as the cutting device was reported to improve the yield of intact plastids isolated from barley leaves (Kannangara _et al._, 1977).

The decline in specific activity on a protein basis for the normal between days 4 to 8 and for the mutant between days 6 and 8 probably represents a true decline since the major chloroplast soluble protein RUBP carboxylase showed minor increases during these stages (Barankiewicz _et al._, 1979) and a selective loss of chloroplast soluble protein is not likely. Inhibitor build-up as the seedlings reached maturity was not found when normal and mutant stromal isolates from seedlings of the same age but different physiological stages were pooled (Table XIII). However an inhibitory effect was evident when 9 day and 1 day whole tissue homogenates of the normal were compared (Table XIII). The assay for the whole tissue comparisons however did not contain KCl, DTT and BSA which were present in the stromal preparations (Table XIII) and it was not determined if the addition of these constituents would relieve the inhibition. Also an increase in activity was not evident upon the addition of an active E. coli carboxyltransferase subunit (Table XII) suggesting a fully functional stromal enzyme complex.

Few studies have been carried out on the possible regulatory role of acetyl CoA carboxylase in fatty acid synthesis in plants. Since activities in this study were determined on the basis of total protein not on purified protein, quantitative changes in the enzyme protein during the maturation of the leaf are not known. The plant enzyme from wheat germ (Burton and Stumpf, 1966), and the E. coli enzyme (Volpe and Vagelos, 1976) are not regulated by the 'feed-forward activators' which include the TCA-cycle intermediates described for animal liver and adipose tissues (Lane et al, 1974). The E. coli system differs from the animal system in that its fatty acids are primarily incorporated into phospholipids which serve as membrane structural components rather than being deposited as a reserve source of energy in the form of triglycerides as is the case in animal liver and adipose tissue (Lane et al, 1974). The regulation of E. coli fatty acid synthesis has been shown to be closely coordinated with the rate of synthesis of macromolecules and cell growth (Volpe and Vagelos, 1976). The chloroplast system also does not form triglycerides (Stumpf,1976) and its acyl lipid synthesis is perhaps coordinated to the rate of cellular development. This appears evident from the acetate labelling studies where an increase in the rate of acyl lipid synthesis corresponded to the period most active in chloroplast development (Hawke et al, 1974; Bolton and Harwood, 1978). Also Murphy and Stumpf (1979) have shown the C_{18}-desaturase activities were selectively induced during the greening of cucumber cotyledons and then fell to much lower levels in the more mature tissue. A link between the regulation of acetyl CoA carboxylase activity and cell growth has been demonstrated for E. coli (Polakis et al 1973). The nucleotides, guanosine 5',3'-diphosphate (ppGpp) and guanosine 5'-diphosphate,3'triphosphate (pppGpp) which accumulate following a nutritional shift-down, were shown to markedly inhibit the carboxyltransferase activity of the acetyl CoA carboxylase reaction (Polakis et al, 1973). It is of interest that when Chlamydomonas reinhardi ac-20 cells were grown under mixotrophic conditions an accumulation of ppGpp was detected and on the shift to autotrophic conditions a reduction in ppGpp synthesis was reported which paralleled the enhanced synthesis of chloroplast ribosomal RNA (Heinzman and Howell, 1978).

The higher biotin content in the stromal fraction relative to the lamellar fraction (Table XVI) is consistent with the localization of the biotin containing enzyme to the stromal fraction (Table VIII). However the much higher levels in the normal relative to the mutant is not consistent with the activities found (Table XI). Also the level of biotin at 1.0 to 3.1 ug/mg protein exceeds that reported for the highly purified barley embryo acetyl CoA carboxylase (0.42 ug/mg protein) (Brock and Kannangara, 1976). It is however somewhat less than the biotin content of the electrophoretically purified BCCP from the barley embryo (10.1 ug/mg protein) (Brock and Kannangara, 1976). The purified E. coli BCCP was found to have a biotin content of 10.7 ug/mg protein (Fall and Vagelos, 1972). The avidin dye-binding method for biotin estimations is of lower sensitivity than the bioassays however it is more precise and convenient to use (Green, 1970; Gyorgy, 1967). The assay is based on the fact that avidin binds stoichiometrically with biotin and the dye (HABA) binds only to avidin (Green, 1970). Binding of HABA to avidin shifts the absorption band from 348 to 500 nm and the dye is displaced by the addition of biotin as shown in Figure 34 (dissociation constants of HABA-avidin and biotin-avidin complexes are 5.8×10^{-6} M and $10x^{-15}$ M respectively (Green, 1970). Biotinyl enzymes will displace the dye when added to the avidin-HABA complex however the reaction is slower than that of free biotin and may not go to completion depending on the complexity of the enzyme (Green, 1970). These problems can be eliminated by digesting the enzyme with pronase (Green,1970). This procedure for biotin estimation was used and presumed adequate for the localization of the acetyl CoA carboxylase since it is the only biotin enzyme described for plants. The high readings obtained probably reflect a high free biotin content in the chloroplast stromal preparations (Table XVI). High levels of soluble biotin were also reported in lettuce, maize, pea, and tobacco chloroplasts (Kannangara and Stumpf, 1973). The assay of non-purified enzyme preparations could also have led to erroneous results since serum albumins are known to bind to avidin (Baxter, 1964) and biotin analogues will also bind to avidin (Green, 1963). The results however do not rule out the possibility that the functional BCCP component of the acetyl CoA carboxylase complex is also associated with the lamellar membrane as was found by others (Kannangara and Stumpf, 1972; Kannangara and Jensen, 1975).

REFERENCES

Alberts, A.W., S.G. Gordon and P.R. Vagelos. 1971. Acetyl CoA carboxylase: The purified transcarboxylase component. Proc. Nat. Acad. Sci. 68: 1259-1263

Alberts, A.W. and P.R. Vagelos. 1968. Acetyl CoA carboxylase, I. Requirement for two protein fractions. Proc. Nat. Acad. Sci. 59: 561-568

Allen, C.F. and P. Good. 1971. Acyl lipids in photosynthetic systems. Methods Enzymol. Vol. 13A: 523-547

Allen, C.F., P. Good, H.F. Davis, P. Chisum and S.D. Fowler. 1966. Methodology for the separation of plant lipids and application to spinach leaf and chloroplast lamellae. J. Am. Oil Chem. Soc. 43: 223-231

Allen, C.F., P. Good, T.S. Trosper and R.B. Park. 1972. Chlorophyll, glycerolipid and protein ratios in spinach chloroplast grana and stroma lamellae. Biochem. Biophys. Res. Commun. 48: 907-913

Amesz, J. 1977. Plastoquinones. *In*: Encyclopedia of Plant Physiology. New Series Photosynthesis I. (A. Trebst and M. Avron, editors). Vol.5: 238-246. Springer-Verlag, Berlin, Heidelberg, New York

Anderson, J.M. 1975. The molecular organization of chloroplast thylakoids. Biochim. Biophys. Acta 416: 191-235

Anderson, J.M. and N.K. Boardman. 1966. Fractionation of the photochemical systems of photosynthesis. I. Chlorophyll content and photochemical activities of particles isolated from spinach chloroplasts. Biochim. Biophys. Acta 112: 403-421

Anderson, J.M., J.C. Waldron and S.W. Thorne. 1978. Chlorophyll-protein complexes of spinach and barley thylakoids. FEBS Lett. 92: 227-233

Apel, K. and K. Kloppstech. 1978. The plastid membranes of barley (*Hordeum vulgare*). Light-induced appearance of mRNA coding for the apoprotein of the light-harvesting chlorophyll a/b protein. Eur. J. Biochem. 85: 581-588

Appelqvist, L.A., J.E. Boynton, P.K. Stumpf and D. von Wettstein. 1968. Lipid biosynthesis in relation to chloroplast development in barley. J. Lipid Res. 9: 425-436

Argyroudi-Akoyunoglou, J.H., Z. Feleki and G. Akoyunoglou. 1972. Photoinduced formation of two different chlorophyll-protein complexes at the early stages of greening. See Boardman *et al*. 1972. Vol. 3: 2417-2426

Arnon, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1-15

Arnon, D.I. 1971. The light reactions of photosynthesis. Proc. Nat. Acad. Sci. 68: 2883-2892

Arnon, D.I. 1977. Photosynthesis 1950-1975: Changing concepts and perspectives. See Amesz, 1977. Vol. 5: 7-56

Arntzen, C.J. 1978. Dynamic structural features of chloroplast lamellae. *In*: Current Topics in Bioenergetics (D.R. Sanadi and L.P. Vernon, editors) Vol. 8B: 111-160. Academic Press, San Francisco, London

Arntzen, C.J. P.A. Armond, J.M. Briantais, J.J. Burke and W.P. Novitrzky. 1977. Dynamic interactions among structural components of the chloroplast membrane. Brookhaven Symp. Biol. 28: 316-337

Bachmann, M.D., D.S. Robertson, C.C. Bowen and I.C. Anderson. 1973. Chloroplast ultrastructure in pigment-deficient mutants of *Zea mays* under reduced light. J. Ultrastruct. Res. 45: 384-406

Bahl, J. 1977. Chlorophyll, carotenoid, and lipid content in *Triticum sativum* L. Plastid envelopes, prolamellar bodies, stroma lamellae and grana. Planta 136: 21-24

Bahl, J., B. Francke and R. Moneger. 1976. Lipid composition of envelopes, prolamellar bodies and other plastid membranes in etiolated, green and greening wheat leaves. Planta 129: 193-201

Bailey, J.L. and A.G. Whyborn. 1963. The osmiophilic globules of chloroplasts. II. Globules of the spinach-beet chloroplast. Biochim.Biophys. Acta 78: 163-174

Baker, N.R. and R.M. Leech. 1977. Development of photosystem I and photosystem II activities in leaves of light-grown maize (*Zea mays*). Plant Physiol. 60: 640-644

Bamberger, E.S. and R.B. Park. 1966. Effect of hydrolytic enzymes on the photosynthetic efficiency and morphology of chloroplasts. Plant Physiol. 41: 1591-1600

Barankiewicz, T.J., R.B. Popovic and S. Zalik. 1979. Ribulose-1 , 5-bisphosphate carboxylase and phosphoenol-pyruvate carboxylase activity in barley and its virescens mutant. Biochem. Biophys. Res. Commun. 87: 884-889

Barr, R., L. Magree and F.L. Crane. 1967. Quinone distribution in horse-chestnut chloroplasts, globules and lamellae. Amer. J. Bot. 54: 365-374

Bartlett, G.R. 1959. Phosphorus assay in column chromatography. J. Biol. Chem. 234: 466-468

Baxter, J.H. 1964. Differences in serum albumins reflected in absorption spectra of a bound dye. Arch. Biochem. Biophys. 108: 375-383

Beale, S.I. 1978. 5-Aminolevulinic acid in plants: Its biosynthesis, regulation and role in plastid development. Ann. Rev. Plant Physiol. 29:95-120

Bedbrook, J.R. and L. Bogorad. 1976. Endonuclease recognition sites mapped on *Zea mays* chloroplast DNA. Proc. Nat. Acad. Sci. 73: 4309-4313

Bedbrook, J.R., G. Link, D. Coen , L. Bogorad and A. Rich. 1978. Maize plastid gene expressed during photoregulated development. Proc. Nat. Acad. Sci. 75: 3060-3064

Bendall, D.S. 1977. Development of photosynthetic electron transport in greening barley. Biochem. Soc. Trans : 84-88

Benson, A.A. 1971. Lipids of Chloroplasts. *In*: Structure and

Function of Chloroplasts (M. Gibbs, editor) pp. 139-148. Springer-Verlag, New York. Heidelberg. Berlin

van Besouw, A. and J.F.G.M. Wintermans. 1978. Galactolipid formation in chloroplast envelopes I. Evidence for two mechanisms in galatosylation. Biochim. Biophys. Acta 529: 44-53

van Besouw, A. and J.F.G. Wintermans. 1979. The synthesis of galactosyldiacylglycerols by chloroplast envelopes. FEBS Lett. 102: 33-37

Bickel, H. and G. Schultz. 1976. Biosynthesis of plastoquinone and β-carotene in isolated chloroplasts. Phytochemistry 15: 1253-1255

Birky, C.W. Jr. 1976. The inheritance of genes in mitochondria and chloroplasts. Bioscience 26: 26-33

Bishop, D.G., K.S. Anderson and R.M. Smillie. 1971. The distribution of galactolipids in mesophyll and bundle sheath chloroplasts of maize and sorghum. Biochim. Biophys. Acta 231: 412-414

Bligh, E.G. and W.S. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917

Bloch, K. and D. Vance. 1977. Control mechanisms in the synthesis of saturated fatty acids. Ann. Rev. Biochem. 46: 263-298

Boardman, N.K. 1970. PhysicaL separation of the photosynthetic photochemical systems. Ann. Rev. Plant Physiol. 21: 115-140

Boardman, N.K. 1977. Development of chloroplast structure and function. See Amesz, 1977. Vol. 5: 583-600

Boardman, N.K. 1977a. Comparative photosynthesis of sun and shade plants. Ann. Rev. Plant Physiol. 28: 355-377

Boardman, N.K., J.M. Anderson and D.J. Goodchild. 1978. Chlorophyll-protein complexes and structure of mature and developing chloroplasts. See Arntzen, 1978. Vol. 8B: 35-109

Boardman, N.K., J.M. Anderson, R.G. Hiller, A. Kahn , P.G. Roughan, T.E. Treffry and S.W. Thorne. 1972. Biosynthesis of the photosynthetic apparatus during chloroplast development in higher plants. In: Proc. II Int. Congr. Photosynthesis (G. Forti, M. Avron and A. Melandri, editors) Vol. 3: 2265-2287. Dr. W. Junk N.V. Publishers, The Hague

Bogorad, L. 1975. Evolution of organelles and eukaryotic genomes. Science 188: 891-898

Bogorad, L. 1975a. Eukaryotic intracellular relationships. In: Membrane Biogenesis, Mitochondria, Chloroplasts and Bacteria (A. Tzagoloff, editor) pp. 201-245. Plenum Press, New York. London

Bogorad, L. 1976. Chlorophyll biosynthesis. In: Chemistry and Biochemistry of Plant Pigments (T.W. Goodwin, editor) Vol. I: 64-148. Academic Press, London, New York, San Francisco

Bolton, P. and J.L. Harwood. 1977. Fatty acid biosynthesis by a particulate prepation from germinating pea.

Biochem. J. 168: 261-269

Bolton, P. and J.L. Harwood. 1978. Fatty acid synthesis by slices from developing leaves. Planta 138: 223-228

Bolton, P., J. Wharfe and J.L. Harwood. 1978. The lipid composition of a barley lacking chlorophyll b. Biochem. J. 174: 67-72

Bottomley, W. and P.R. Whitfeld. 1979. Cell-free transcription and translation of total spinach chloroplast DNA. Eur. J. Biochem. 93: 31-39

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254

Branton, D. 1966. Fracture faces of frozen membranes. Proc. Nat. Acad. Sci. 55: 1048-1056

Branton, D., S. Bullivant, N.B. Gilula, M.J. Karnovsky, H. Moor, K. Muhlethaler, D.H. Northcote, L. Packer, B. Satir, P. Satir, V. Speth, L.A. Staehelin, R.L. Steere and R.S. Weinstein. 1975. Freeze-etching nomenclature. Science 190: 54-56

Britton, G. 1976. Biosynthesis of carotenoids. See Bogorad, 1976. Vol I: 262-327

Brock, K. and C.G. Kannangara. 1976. Properties and morphology of barley embryo acetyl CoA carboxylase. Carlsberg Res. Commun. 41: 121-129

Brooks, J.L. and P.K. Stumpf. 1966. Fat metabolism in higher plants. XXXIX. Properties of a soluble fatty acid synthesizing system from lettuce chloroplasts. Arch. Biochem. Biophys. 116: 108-116

Burden, T.S. and D.T. Canvin. 1975. The effect of Mg on the density of proplastids from the developing castor bean and the use of acetyl-CoA carboxylase activity for their cytochemical identification. Can. J. Bot. 53: 1371-1376

Burton, D. and P.K. Stumpf. 1966. Fat metabolism in higher plants XXXII. Control of plant acetyl-CoA carboxylase activity. Arch. Biochem. Biophys. 117: 604-614

Butterfass, T. 1973. Control of plastid division by means of nuclear DNA amount. Protoplasma 76: 167-195

Clayton, R.K. 1963. Photosynthesis: primary physical and chemical processes. Ann. Rev. Plant Physiol. 14: 159-180

Coen, D.M., J.R. Bedbrook, L. Bogorad and A. Rich. 1977. Maize chloroplast DNA fragment encoding the large subunit of ribulosebisphosphate carboxylase. Proc. Nat. Acad. Sci. 74: 5487-5491

Davies, B.H. 1976. Carotenoids. See Bogorad, 1976. Vol. 2: 38-165.

Davies, B.H. 1977. Carotenoids in higher plants. In: Lipids and Lipid Polymers in Higher Plants (M. Tevini and H.K. Lichtenthaler, editors) pp. 199-217. Springer-Verlag, Berlin, Heidelberg, New York

Denison, F.W. Jr. and E.F. Phares. 1952. Rapid method for paper chromatography of organic acids. Anal. Chem. 24: 1628-1629

Devor, K.A. and J.B. Mudd. 1971. Biosynthesis of phosphatidylcholine by enzyme preparation from spinach leaves. J. Lipid Res. 12: 403-411

Dickman, S.R. and A.L. Crockett. 1956. Reactions of xanthydrol. IV Determination of tryptophan in blood plasma and in proteins. J.Biol. Chem. 220: 957-965

Dittmer, J.C. and R.L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. 5: 126-127

Douady, D., J.C. Kader and P. Mazliak. 1978. Properties of plant phospholipid-exchange proteins. Phytochemistry 17: 793-794

Douce, R. 1974. Site of biosynthesis of galactolipids in spinach chloroplasts. Science 183: 852-853

Douce, R., R.B. Holtz and A.A. Benson. 1973. Isolation and properties of the envelope of spinach chloroplasts. J. Biol. Chem. 248: 7215-7222

Downey, R.K. and D.I. McGregor. 1976. Breeding for modified fatty acid composition. In: Commentaries in Plant Science (H. Smith, editor) pp. 153-169. Pergamon Press, Oxford

Dubacq, J.P., D. Mazliak and A. Tremolieres. 1976. Sub-cellular localization of the oleyl-Co A desaturase activity in pea leaves. FEBS Lett. 66: 183-186

Egger, K. 1969. Plant phenol derivatives. In: Thin-Layer Chromatography (E. Stahl, editor) pp. 687-706. Springer-Verlag, New York

Eisenberg, M.A. 1973. Biotin: biogenesis, transport, and their regulation. Advances Enzymology 38: 317-372

Ellis, J.R. 1976. Fraction I Protein. See Downey and McGregor, 1976. pp. 31-43

Ellis, R.J. 1977. Protein synthesis by isolated chloroplasts. Biochim. Biophys. Acta 463: 185-215

Fall, R.R. and P.R. Vagelos. 1972. Acetyl coenzyme A carboxylase. Molecular forms and subunit composition of biotin carboxyl carrier protein. J. Biol. Chem. 247: 8005-8015

Folch, J., M. Lees and G.H.S. Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497-509

Freeman, B.A., K. Platt-Aloia, J.B. Mudd and W.W. Thomson. 1978. Ultrastructural and lipid changes associated with the aging of citrus leaves. Protoplasma 94: 221-233

Gerwin, B.I., B.E. Jacobson and H.G. Wood. 1969. Transcarboxylase, VIII. Isolation and properties of a biotin-carboxyl carrier protein. Proc. Nat. Acad. Sci. 64: 1315-1322

Gillham, N.W. 1974. Genetic analysis of the chloroplast and mitochondrial genomes. Ann. Rev. Genet. 8: 347-391

Gillham, N.W., J.E. Boynton and N.H. Chuas. 1978. Genetic control of chloroplast proteins. See Arntzen, 1978. Vol. 8B: 211-260

Givan C.V. and J.L. Harwood. 1976. Biosynthesis of small molecules in chloroplasts of higher plants. Biol. Rev. 51: 365-406

Goodwin, T.W. 1976. Distribution of carotenoids. See Bogorad, 1976. Vol. 1: 225-261

Goodwin, T.W. 1977. The prenylipids of the membranes of higher plants. See Davies, 1977. pp. 29-47

Gough, S.P. 1978. Light stimulated 5-aminolevulinate accumulation in levulinate treated barley seedlings. Carlsberg Res. Commun. 43: 497-5087

Gough, S.P. and C.G. Kannangara. 1976. Synthesis of 5-ALA by isolated plastids, Carlsberg Res. Commun. 41: 183-190

Gough, S.P. and C.G. Kannangara. 1977. Synthesis of 5-aminolevulinate by a chloroplast stroma preparation from barley leaves. Carlsberg Res. Commun. 42: 459-464

Granick, S. and K.R. Porter. 1947. The structure of the spinach chloroplast as interpreted with electron microscope. Am. J. Bot. 34: 545-550

Grebanier, A.E., K.E. Steinbeck and L. Bogorad. 1979. Comparison of the molecular weights of proteins synthesized by isolated chloroplasts with those which appear during greening in *Zea mays*. Plant Physiol. 63: 436-439

Green, N.M. 1963. Avidin. The nature of the biotin binding site. Biochem. J. 89: 599-609

Green, N.M. 1970. Spectrophotometric determination of avidin and biotin. Methods Enzymol. 18A: 418-424

Greenwood, A.D., R.M. Leech and J.P. Williams. 1963. The osmiophilic globules of chloroplasts. I. Osmiophilic globules as a normal component of chloroplasts and their isolation and composition in *Vicia faba* L. Biochim. Biophys. Acta 78: 148-162

Griffiths, W.T. 1974. Protochlorophyll and protochlorophyllide as precursors for chlorophyll synthesis in vitro. FEBS Lett. 49: 196-200

Grun, P. 1976. Cytoplasmic Genetics and Evolution. pp. 85-165. Columbia University Press, New York

Gyorgy, P. 1967. Biotin. *In*: The Vitamins. (P. Gyorgy and W.N. Pearson, editors) vol. 7: 303-313. Academic Press, New York, London

Harwood, J.L. 1975. Fatty acid biosynthesis. *In*: Recent Advances in the Chemistry and Biochemistry of Plant Lipids (T. Galliard and E.I. Mercer, editors) pp. 44-113. Academic Press, London, New York, San Francisco

Harwood, J.L. 1975a. Synthesis of sulphoquinovosyl diacylglycerol by higher plants. Biochim. Biophys. Acta 398: 224-230

Harwood, J.L. and A.T. James. 1975. Metabolism of trans-3-hexadecenoic acid in broad bean. Eur. J. Biochem. 50: 325-334

Harwood, J.L. and R.G. Nicholls. 1979. The plant sulpholipid a major component of the sulphur cycle. Biochem. Soc. Trans. 7: 440-447

Harwood, J.L. and P.K. Stumpf. 1976. Plant acyl lipids: evolutional curiosities or functional constituents? Trends Biochem. Sci. 1: 253-256

Hatch, M.D. and P.K. Stumpf. 1961. Fat Metabolism in Higher

Plants. XVI. Acetyl coenzyme A carboxylase and acyl coenzyme A - malonyl coenzyme A transcarboxylase from wheatgerm. J. Biol. Chem. 236: 2879-2885

Haverkate, F. and L.L.M. Van Deenen. 1965. Isolation and chemical characterization of phosphatidyl glycerol from spinach leaves. Biochim. Biophys. Acta 106: 78-92

Hawke, J.C, M.G. Rumsby and R.M. Leech. 1974. Lipid biosynthesis in green leaves of developing maize. Plant Physiol. 53: 555-561

Hawke, J.C., M.G. Rumsby and R.M. Leech. 1974a. Lipid biosynthesis by chloroplasts isolated from developing Zea mays. Phytochemistry 13: 403-413

Heinstein, P.F. and P.K. Stumpf. 1969. Fat metabolism in higher plants. XXXVIII. Properties of wheat germ acetyl coenzyme A carboxylase. J. Biol. Chem. 244: 5374-5381

Heinz, E. 1977. Enzymatic reactions in galactolipid biosynthesis. See Davies, 1977. pp. 102-120

Heinz, E. and J.L. Harwood. 1977. Incorporation of carbon dioxide, acetate and sulphate into the glycerolipids of Vicia faba leaves. Hoppe-Seyler's Z. Physiol. Chem. 358: 897-908

Helenius, A. and K. Simons. 1975. Solubilization of membranes by detergents. Biochim. Biophys. Acta 415: 29-79

Henningsen, K.W. and J.E. Boynton. 1974. Macromolecular physiology of plastids. IX. Development of plastid membranes during greening of dark-grown barley seedlings. J. Cell Sci. 15: 31-55

Henningsen, K.W. and A. Kahn. 1971. Photoactive subunits of protochlorophyllide holochrome. Plant Physiol. 47: 685-690

Henriques, F. and R.B. Park 1978. Spectral characterization of five chlorophyll-protein complexes. Plant Physiol. 62: 856-860

Hernandez-Lucuz, C., R.F. DeCaleya, P. Carbonero and F. Garcia-Olmedo. 1977. Control of galactosyl diglycerides in wheat endosperm by group 5 chromosomes. Genetics 85: 521-527

Hirayama, O. and T. Nomotobori. 1978. Preparation and characterization of phospholipid depleted chloroplasts. Biochim. Biophys. Acta 502: 11-16

Hitchcock, C. and B.W. Nichols. 1971. Plant Lipid Biochemistry. Academic Press, London, New York

Horak, A. and R.D. Hill. 1971. Coupling factor for photophosphorylation in bean etioplasts and chloroplasts. Can. J. Biochem. 49: 207-209

Horak, A. and S. Zalik. 1975. Development of photoreductive activity in plastids of a virescens mutant of barley. Can. J. Bot. 53: 2399-2404

Huang, K.P. 1970. A sensitive assay method of acetyl CoA synthetase. Anal. Biochem. 37: 98-104

Hughes, D.E., J.W.T. Wimpenny and D. Lloyd. 1971. The disintegration of micro-organisms. In: Methods in Microbiology (J.R. Norris and D.W. Ribbons, editors)

Vol. 5B: 1-54. Academic Press, New York, London

van Hummel, H.C., Th. J.M. Hulsebos and J.F.G.M. Wintermans. 1975. Biosynthesis of galactosyl diglycerides by non-green fractions from chloroplasts. Biochim. Biophys. Acta 380: 219-226

Huzisige, H., H. Usiyama, T.K. Kuti and T. Azi. 1969. Purification and properties of the photoactive particle corresponding to Photosystem II. Plant and Cell Physiol. 10: 441-455

Izawa, S. and N.E. Good. 1966. Effect of salts and electron transport on the conformation of isolated chloroplasts. II. Electron microscopy. Plant Physiol. 41: 544-552

Jacobi, G. 1977. Subchloroplast preparations. See Amesz, 1977. Vol. 5: 543-562

Jacobs, S. 1965. The determination of nitrogen in biological materials. In: Methods of Biochemical Analysis (D. Glick, editor) Vol. 13: 241-263

Jacobson, B.S., C.G. Kannangara and P.K. Stumpf. 1973. The elongation of medium chain trienoic acids to α-linolenic acid by a spinach chloroplast stroma system. Biochem. Biophys. Res. Commun. 52: 1190-1198

James, A.T. and B.W. Nichols. 1966. Lipids of photosynthetic systems. Nature 210: 372-375

Jeffrey, S.W. 1968. Quantitative thin-layer chromatography of chlorophylls and carotenoids from marine algae. Biochim. Biophys. Acta 162: 271-285

Jeffrey, S.W., R. Douce and A.A. Benson. 1974. Carotenoid transformations in the chloroplast envelope. Proc. Nat. Acad. Sci. U.S.A. 71: 807-810

Jhamb, S. and S. Zalik. 1973. Soluble and lamellar proteins in seedlings of barley and its virescens mutant in relation to chloroplast development. Can. J. Bot. 51: 2147-2154

Jhamb, S. and S. Zalik. 1975. Plastid development in a virescens barley mutant and chloroplast microtubules. Can. J. Bot. 53: 2014-2025

Joyard, J. and R. Douce. 1977. Site of synthesis of phosphatidic acid and diacylglycerol in spinach chloroplasts. Biochim. Biophys. Acta 486: 273-285

Jurd, L. 1962. Spectral properties of flavonoid compounds. In: The Chemistry of Flavonoid Compounds (T.A. Geissman, editor) pp. 107-155. Pergamon Press, New York, Oxford

Kader, J.C. 1975. Proteins and the intracellular exchange of lipids. I. Stimulation of phospholipid exchange between mitochondria and microsomal fractions by proteins isolated from potato tuber. Biochim. Biophys. Acta 380: 31-44

Kahn, A., N. Avivi-Bleiser and D. von Wettstein. 1976. Genetic regulation of chlorophyll synthesis analyzed with double mutants in barley. In: Genetics and Biogenesis of Chloroplasts and Mitochondria (Th. Bucher, W. Newport, W. Sebald and S. Werner, editors) pp. 119-131. North-Holland Publishing Co., Amsterdam, New York, Oxford

Kaldewey, H. 1969. "Simple" indole derivatives and plant growth regulators. See Egger, 1969. pp. 471-493

Kannangara, C.G., B.S. Jacobson and P.K. Stumpf. 1973. Fat metabolism in higher plants LVII. A comparison of the fatty acid synthesizing enzymes in chloroplasts isolated from mature and immature leaves of spinach. Plant Physiol. 52: 156-161

Kannangara, C.G. and S.P. Gough. 1977. Synthesis of 5-aminolevulinic acid and chlorophyll by isolated chloroplasts. Carlsberg Res. Commun. 42: 441-457

Kannangara, C.G. and S.P. Gough. 1979. Biosynthesis of 5-aminolevulinate in greening barley leaves. II. Induction of enzyme synthesis by light. Carlsberg Res. Commun. 44: 11-20

Kannangara, C.G., S. Gough, B. Hansen, J.N. Rasmussen and D.J. Simpson. 1977. A homogenizer with replaceable razor blades for bulk isolation of active barley plastids. Carlsberg Res. Commun. 42: 431-439

Kannangara, C.G., K.W. Henningsen, P.K. Stumpf, L.A. Appelqvist and D. von Wettstein. 1971. Lipid biosynthesis by isolated barley chloroplasts in relation to plastid development. Plant Physiol. 48: 526-531

Kannangara, C.G., K.W. Henningsen, P.K. Stumpf and D. von Wettstein. 1971a. 6-Methylsalicyclic acid synthesis by isolated barley chloroplasts. Eur. J. Biochem. 21: 334-338

Kannangara, C.G. and C.J. Jensen. 1975. Biotin carboxyl carrier protein in barley chloroplast membranes. Eur. J. Biochem. 54: 25-30

Kannangara, C.G. and P.K. Stumpf. 1972. Fat metabolism in higher plants. LIV. A procaryotic type acetyl CoA carboxylase in spinach chloroplasts. Arch. Biochem. Biophys. 152: 83-91

Kannangara, C.G. and P.K. Stumpf. 1973. Fat metabolism in higher plants. LVI. Distribution and nature of biotin in chloroplasts of different plant species. Arch. Biochem. Biophys. 155: 391-399

Kates, M. 1970. Plant Phospholipids and glycolipids. In: Advances in Lipid Research (R. Paoletti and D. Kritschevsky, editors) Vol. 8: 225-265. Academic Press, New York, London

Kates, M. and M.O. Marshall. 1975. Biosynthesis of phosphoglycerides in plants. See Harwood, 1975. pp. 115-159

Katoh, S. 1977. Plastocyanin. See Amesz, 1977. Vol. 5: 247-252

Ke, B., 1971. Carotenoproteins. Methods Enzymol. Vol. 23A: 624-636

Keck, R.W., R.A. Dilley, C.F. Allen and S. Biggs. 1970. Chloroplast composition and structure differences in a soybean mutant. Plant Physiol. 46: 692-698

Kirk, J.T.O. 1967. Parts I and III. In: The Plastids, Their Chemistry, Structure, Growth and Inheritance (By J.T.O. Kirk and R.A.E. Tilney-Basset). W.H. Freeman and Co., London, San Francisco

Kirk, J.T.O. 1971. Chloroplast structure and biogenesis. Ann. Rev. Biochem. 40: 161-196

Kirk, J.T.O. 1971a. Will the real chloroplast DNA please stand up. In: Autonomy and Biogenesis of Mitochondria and Chloroplasts. (N.K. Boardman, A.W. Linnane and R.M. Smillie, editors) pp. 267-276. North-Holland Publishing Co., Amsterdam, London

Kleinig, H. and B. Liedvogel. 1979. On the energy requirements of fatty acid synthesis in spinach chloroplasts in the light and in the dark. FEBS Lett. 101: 339-342

Kolattukudy, P.E. 1977. Biosynthesis and degradation of lipid polymers. See Davies, 1977. pp. 271-292

Krupa, Z. and T. Baszynski. 1975. Requirement of galactolipids for photosystem I activity in lyophilized spinach chloroplasts. Biochim. Biophys. Acta 408: 26-34

Kuksis, A. 1966. Quantitative lipid analysis by combined thin layer and gas liquid chromatographic systems. Chromatogr. Rev. 8: 172-207

Kung, S. 1977. Expression of chloroplast genomes in higher plants. Ann. Rev. Plant Physiol. 238: 401-437

Laetsch, W.M. 1974. The C_4 Syndrome: A structural analysis. Ann. Rev. Plant Physiol. 25: 27-52

Lane, M.D., J. Moss and S.E. Polakis. 1974. Acetyl coenzyme A carboxylase. In: Current Topics in Cellular Regulation. (B.L. Horecker and E.R. Stadtman, editors). Vol. 8: 139-195

Larsson, C., B. Andersson and G. Roos. 1977. Scanning electron microscopy of different populations of chloroplasts isolated by phase partition. Plant Sci. Lett. 8: 291-298

Leech, R.M. 1976. Plastid development in isolated etiochloroplasts and isolated etioplasts. In: Perspectives in Experimental Biology (N. Sunderland, editor) Vol. 2: 145-162. Pergamon Press, Oxford, New York

Leech, R.M. and D.J. Murphy. 1976. The cooperative function of chloroplasts in the biosynthesis of small molecules. In: Topics in Photosynthesis. The Intact Chloroplast (J. Barber, editor) Vol. 1: 365-401. Elsevier Scientific, Amsterdam, New York, Oxford

Leech, R.M., M.G. Rumsby and W.W. Thomson. 1973. Plastid differentiation, acyl lipid and fatty acid changes in developing green maize leaves. Plant Physiol. 52: 240-245

Leese, B.M. and R.M. Leech. 1976. Sequential changes in the lipids of developing proplastids isolated from green maize leaves. Plant Physiol. 5: 789-794

Lichtenthaler, H.K. 1969. Localization and functional concentrations of lipoquinones in chloroplasts. In: Progress in Photosynthesis Research (H. Metzner, editor) Vol. 1: 304-314

Lichtenthaler, H.K. 1969b. Die Plastoglobuli von Spinat, ihre Groẞe und Zusammensetzung wahrend der Chloroplastendegeneration (English abstract) Protoplasma 68: 315-326

Lichtenthaler, H.K. 1977. Regulation of prenylquinone synthesis in higher plants. See Davies, 1977: pp. 231-258

Lichtenthaler, H.K. and R.B. Park. 1963. Chemical composition of chloroplast lamellae from spinach. Nature 198: 1070-1072

Liljenberg, C. 1977. Chlorophyll formation: the phytylation step. See Davies, 1977: pp. 257-270

Lilley, R.M., M.P. Fitzgerald, K.G. Rienits and D.A. Walker. 1975. Criteria of intactness and the photosynthetic activity of spinach chloroplast preparations. New Phytol. 75: 1-10

Lord, J.M. 1976. Phospholipid synthesis and exchange in castor bean endosperm homogenates. Plant Physiol. 57: 218-223

Lord, J.M. 1978. Evidence that a proliferation of the endoplasmic reticulum precedes the formation of glyoxysomes and mitochondria in germinating castor bean endosperm. J. Exp. Bot. 29: 13-23

Lowry, O.H., N.J. Rosebough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275

Mabry, T.J., K.R. Markham and M.B. Thomas. 1970. The Systematic Identification of Flavonoids. pp. 3-15. Springer-Verlag, New York, Heidelberg, Berlin

Macher, B.A. and J.B. Mudd. 1974. Biosynthesis of phosphatidylethanolamine by enzyme preparations from plant tissue. Plant Physiol. 53: 171-175

Machold, O., D.J. Simpson and G. Hoyer-Hansen. 1977. Correlation between the freeze fracture appearance and polypeptide composition of thylakoid membrane in barley. Carlsberg Res. Commun. 42: 499-516

Maclachlan, S. 1962. Microscopic and Biochemical Studies on a Chlorophyll Mutant of Gateway Barley. Ph.D. Thesis university of Alberta, Edmonton

Maclachlan, S. and S. Zalik. 1963. Plastid structure, chlorophyll concentration, and free amino acid composition of a chlorophyll mutant of barley. Can. J. Bot. 41: 1053-1062

Mackender, R.O. 1978. Etioplast development in dark-grown leaves of *Zea mays* L. Plant Physiol. 62: 499-505

Mackender, R.O. and R.M. Leech. 1974. The galactolipid, phospholipid and fatty acid composition of the chloroplast envelope membranes of *Vicia faba* L. Plant Physiol. 53: 496-502

Markham, K.R. and T.J. Mabry. 1975. Ultraviolet visible and proton magnetic resonance spectroscopy of flavonoids. *In*: The Flavonoids (J.B. Harborne, T.J. Mabry and H. Mabry, editors) pp. 45-61. Chapman and Hall, London

Markwell, J.P., J.P. Thornber and R.T. Boggs. 1979. Higher plant chloroplasts: Evidence that all the chlorophyll exists as chlorophyll-protein complexes. Proc. Nat. Acad. Sci. 76: 1233-1235

Marshall, M.O. and M. Kates. 1974. Biosynthesis of nitrogenous phospholipids in spinach leaves. Can. J. Biochem. 52: 469-482

Mazliak, P. 1977. Glyco and phospholipids of biomembranes in higher plants. See Davies, 1977. pp. 48-74

McMahon, D. 1975. Cycloheximide is not a specific inhibitor of protein synthesis *in vivo*. Plant Physiol. 55: 815-821

Miller, A.L. and H.R. Levy. 1969. Rat mammary acetyl coenzyme A carboxylase I. Isolation and characterization. J. Biol. Chem. 244: 2334-2342

Miller, R.A. 1965. Physiological and Biochemical Studies of a Mutant from Gateway Barley. Ph.D. Thesis, University of Alberta, Edmonton

Mitchell, H.L. 1971. Microdetermination of nitrogen in plant tissues. J.A.O.A.C. 55: 1-3

Moore, R.M., J.D. Williams and J. Chia. 1967. Factors affecting concentrations of dimethylated indole alkylamines in *Phalaris tuberosa* L. Aust. J. Biol. Sci. 20: 1131-1140

Mudd, J.B. and R.E. Garcia. 1975. Biosynthesis of glycolipids. See Harwood, 1975: pp. 161-201

Mudd, J.B. and T.T. McManus. 1962. Metabolism of acetate by cell-free preparations from spinach leaves. J. Biol. Chem. 237: 2057-2063

Muhlethaler, K. 1971. The ultrastructure of plastids. See Benson, 1971. pp. 5-34

Muhlethaler, K. 1977. Introduction to structure and function of the photosynthetic apparatus. See Amesz, 1977. Vol. 5: 503-521

Murphy, D.J. and R.M. Leech. 1977. Lipid biosynthesis from ^{14}C bicarbonate, 2-^{14}C pyruvate and 1-^{14}C acetate during photosynthesis by isolated spinach chloroplasts. FEBS Lett. 77: 164-168

Murphy, D.J. and R.M. Leech. 1978. The pathway of ^{14}C-bicarbonate incorporation into lipids in isolated photosynthesizing spinach chloroplasts. FEBS Lett. 88: 192-196

Murphy, D.J. and P.K. Stumpf. 1979. Light-dependent induction of polyunsaturated fatty acid biosynthesis in greening cucumber cotyledons. Plant Physiol. 63: 328-335

Nakamura, Y. and M. Yamada. 1974. Long chain fatty acid synthesis in developing castor bean seeds I. Plant Cell Physiol. 15: 37-48

Nozaki, M. and O. Hayaishi. 1971. Separation and purification of proteins. Methods Microbiol. Vol. 5B: 425-454

Nolan, W.G. and R.B. Park. 1975. Comparative studies on the polypeptide composition of chloroplast lamellae and lamellar fractions. Biochim. Biophys. Acta 375: 406-421

Ogawa, T., F. Obata and K. Shibata. 1966. Two pigment proteins in spinach chloroplasts. Biochim. Biophys. Acta 112: 223-234

Ohad, I. 1975. Biogenesis of chloroplast membranes. See. Bogorad, 1975a: pp. 279-350

Ohlrogge, J.B., D.N. Kuhn and P.K. Stumpf. 1979. Subcellular localization of acyl carrier protein in leaf protoplasts of *Spinacia oleracea*. Proc. Nat. Acad. Sci. 76: 1194-1198

Ohlrogge, J.B., W.E. Shine and P.K. Stumpf. 1978. Fat metabolism in higher plants. Characterization of plant acyl-ACP and acyl-CoA hydrolases. Arch. Biochem. Biophys. 189: 382-391

Ojakian, G.K. and P. Satir. 1974. Particle movements in chloroplast membranes: Quantitative measurements of membrane fluidity by the freeze-fracture technique. Proc. Nat. Acad. Sci. 71: 2052-2056

Ongun, A., W.W. Thomson and J.B. Mudd. 1968. Lipid composition of chloroplasts isolated by aqueous and nonaqueous techniques. J. Lipid Res. 9: 409-415

Panter, R.A. and N.K. Boardman. 1973. Lipid biosynthesis by isolated plastids from greening pea, *Pisum sativum*. J. Lipid Res. 14: 664-671

Park, R.B. and N.G. Pon. 1963. Chemical composition and the substructure of lamellae isolated from *Spinacea oleracea* chloroplasts. J. Mol. Biol. 6: 105-114

Park, R.B. and P.V. Sane. 1971. Distribution of function and structures in chloroplast lamellae. Ann. Rev. Plant Physiol. 22: 395-430

Plesnicar, M. and D.S. Bendall. 1973. The photochemical activities and electron carriers of developing barley leaves. Biochem. J. 136: 803-812

Poincelot, R.P. 1973. Isolation and lipid composition of spinach chloroplast envelope membranes. Arch. Biochem. Biophys. 159: 134-142

Polakis, S.E. R.B. Guchhait and M.D. Lane. 1973. Stringent control of fatty acid synthesis in *E. coli*. Possible regulation of acetyl coenzyme A carboxylase by ppGpp. J. Biol. Chem. 248: 7957-7966

Quail, P.H. 1979. Plant cell fractionation. Ann. Rev. Plant Physiol. 30: 425-484

Rebeiz, C.A., S. Larson, T.E. Weier and P.A. Castelfranco. 1973. Chloroplast maintenance and partial differentiation *in vitro*. Plant Physiol. 51: 651-659

Reid, E.E., P. Thomson, C.R. Lyttle and D.T. Dennis. 1977. Pyruvate dehydrogenase complex from higher plant mitochondria. Plant Physiol. 59: 842-848

Reitzel, L. and N.C. Nielsen. 1976. Acetyl-CoA carboxylase during development of plastids in wild-type and mutant barley seedlings. Eur. J. Biochem. 65: 131-138

Robertson, D. and W.M. Laetsch. 1974. Structure and function of developing barley plastids. Plant Physiol. 54: 148-159

Rohmer, M., P. Bouvier and G. Ourisson. 1979. Molecular evolution of biomembranes: Structural equivalents and phylogenetic precursors of sterols. Proc. Nat. Acad. Sci. 76: 847-851

Roughan, P.G. and R.D. Batt. 1968. Quantitative analysis of sulfolipid (sulfoquinovosyl diglyceride) and galactolipids (monogalactosyl and digalactosyl diglyceride) in plant tissues. Anal. Biochem. 22: 74-88

Roughan, P.G. and N.K. Boardman. 1972. Lipid composition of pea and bean leaves during chloroplast development. Plant Physiol. 50: 31-34

Roughan, P.G. and C.R. Slack. 1977. Long-chain acyl-coenzyme A synthetase activity of spinach chloroplast is concentrated in the envelope. Biochem. J. 162: 457-459

Roughan, P.G., C.R. Slack and R. Holland. 1976. High rates of 1-^{14}C acetate incorporation into the lipid of isolated spinach chloroplasts. Biochem. J. 158: 593-601

Sager, R. 1972. Cytoplasmic Genes and Organelles. Chap 1-3. Academic Press, New York, London

Sandermann, H. 1978. Regulation of membrane enzymes by lipids. Biochim. Biophys. Acta 515: 209-237

Sane, P.V. and S. Zalik. 1970. Metabolism of acetate-2-^{14}C, glycine 2-^{14}C and leucine-U-^{14}C , and effect of 5 aminolevulinic acid on chlorophyll synthesis in Gateway barley and its mutant. Can. J. Bot. 48: 1171-1178

Schneider, E.A., R.A. Gibson and F. Wightman. 1972. Biosynthesis and metabolism of indol-3yl-acetic acid. The native indoles of barley and tomato shoots. J. Exp. Bot. 23: 152-170

Schwertner, H.A. and J.B. Biale. 1973. Lipid composition of plant mitochondria and of chloroplasts. J. Lipid Res. 14: 235-242

Sellden, G. and E. Selstam. 1976. Changes in chloroplast lipids during the development of photosynthetic activity in barley etio-chloroplasts. Physiol. Plant 37: 35-41

Shaw, A.B., M.M. Anderson and R.E. McCarty. 1976. Role of galactolipids in spinach chloroplast lamellar membranes. II. Effect of galactolipid depletion on phosphorylation and electron flow. Plant Physiol. 57: 724-729

Sherratt, D. and C.V. Givan. 1973. The apparent absence of a pathway for synthesis of acetyl coenzyme A in pea chloroplasts: Lack of $^{14}CO_2$ incorporation into lipids by the isolated organelle. Planta 113: 47-52

Shibata, K. 1957. Spectroscopic studies on chlorophyll formation in intact leaves. J. Biochem. 44: 147-173

Simcox, P.D., E.E. Reid, D.T. Canvin and D.T. Dennis. 1977. Enzymes of the glycolytic and pentose phosphate pathways in proplastids from the developing endosperm of Ricinus communis L. Plant Physiol. 59: 1128-1132

Simpson, E.E. and J.P. Williams. 1979. Galactolipid synthesis in Vicia faba leaves. IV. Site(s) of fatty acid incorporation into the major glycerolipids. Plant Physiol. 63: 674-676

Singer, S.J. and G.L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. Science 175: 720-731

Sironval, C., J.M. Michel, R. Bronchart and E. Englert-Dujardin. 1969. On the "primary" thylakoids of chloroplasts grown under a flash regime. Prog.

Photosynthesis Res. (H. Metzner, editor) Vol. 1: 47-59

Skipski, V.P. and M. Barclay. 1969. Thin-layer chromatography of lipids. Methods Enzymol. 14: 530-598

Slack, C.R. and P.G. Roughan. 1975. The kinetics of incorporation in vivo of ^{14}C acetate and ^{14}C carbon dioxide into the fatty acids of glycerolipids in developing leaves. Biochem. J. 152: 217-228

Slack, C.R., P.G. Roughan and N. Balasingham. 1977. Labelling studies in vivo on the metabolism of the acyl and glycerol moieties of the glycerolipids in the developing maize leaf. Biochem. J. 162: 289-296

Slack, C.R., P.G. Roughan and J. Terpstra. 1976. Some properties of a microsomal oleate desaturase from leaves. Biochem. J. 155: 71-80

Smillie, R.M., N.C. Nielsen, K.W. Henningsen and D. von Wettstein. 1975. Proc. 3rd Int. Congress on Photosynthesis (M. Avron, editor) pp. 1841-1860. Elsevier Scientific Publishing Company, Amsterdam, Oxford, New York

Smirnov, B.P. 1960. Fatty acid synthesis from acetate by spinach chloroplasts. Biokhimiya 25: 419-426

Smith, B.B. and C.A. Rebeiz. 1979. Chloroplast biogenesis. XXIV. Intrachloroplastic localization of the biosynthesis and accumulation of protoporphyrin IX, magnesium-protoporphyrin monoester, and longer wavelength metalloporphyrins during greening. Plant Physiol. 63: 227-231

Stephansen, K. and S. Zalik. 1971. Inheritance and qualitative analysis of pigments in a barley mutant. Can. J. Bot. 49: 49-51

Stubbe, W. 1971. Origin and continuity of plastids. In: Origin and Continuity of Cell Organelles. pp. 65-81. (J. Reinert and H. Ursprung, editors) Springer-Verlag, New York, Heidelberg, Berlin

Stumpf, P.K. 1975. Biosynthesis of fatty acids in spinach chloroplasts. See Harwood, 1975: 95-113

Stumpf, P.K. 1976. Membrane-bound enzymes in plant lipid metabolism. In: The Enzymes of Biological Membranes (A. Martonosi, editor) Vol. 2: 145-159

Stumpf, P.K. 1976a. Lipid metabolism. In: Plant Biochemistry (J. Bonner and J.E. Varner, editors) pp. 427-461. Academic Press, New York, San Francisco, London

Stumpf, P.K. 1977. Lipid biosynthesis in higher plants. In: Internat. Rev. Biochem. Biochemistry of Lipids II (T.W. Goodwin, editor) Vol. 14: 215-237

Stumpf, P.K. and A.T. James. 1963. The biosynthesis of long chain fatty acids by lettuce chloroplast preparations. Biochim. Biophys. Acta 70: 20-32

Stummann, B.M. 1978. The capacity of various detergents to solubilize and stabilize protochlorophyll(ide) holochrome. Physiol. Plant 43: 173-176

Swain, T. 1976a Flavonoids. See Bogorad, 1976, Vol. 2: 166-206

Swain, T. 1976b. Nature and properties of flavonoids. See

Bogorad, 1976, Vol. 1: 425-463

Swanson, E.S., W.W. Thomson and J.B. Mudd. 1973. The effect of lipid extraction on electron microscopic images of plant membranes. Can. J. Bot. 51: 1221-1229

Tevini, M. 1972. The formation of lipids following illumination of etiolated seedlings. In:Proc. IInd International Congress of Photosynthesis. (G. Forti, M. Avron and A. Melandri, editors) pp. 2471-2478. Dr. W. Junk N.V. Publishers. The Hague

Thomson, L.W. and S. Zalik. 1973. Lipids in rye seedlings in relation to vernalization. Plant Physiol. 52: 268-273

Thornber, J.P. 1975. Chlorophyll-proteins: light-harvesting and reaction center components of plants. Ann. Rev. Plant Physiol. 26: 127-158

Thornber, J.P., R.P.F. Gregory, C.A. Smith and J.L. Bailey. 1967. Studies on the nature of the chloroplast lamella. I. Preparation and some properties of two chlorophyll-protein complexes. Biochem. 6: 391-396

Thornber, J.P., J.P. Markwell and S. Reinman. 1979. Plant chlorophyll-protein complexes: recent advances. Photochem. Photobiol. 29: 1205-1216

Tilney-Bassett, R.A.E. 1975. Genetics of variegated plants. In Genetics and Biogenesis of Mitochondria and Chloroplasts (C.W. Birky, P.S. Perlman and T.J. Byers, editors) pp. 268-308. Ohio State University Press, Columbus

Tobin, E.M. 1978. Light regulation of specific mRNA species in Lemma gibba L. G-3. Proc. Nat. Acad. Sci. 75: 4749-4753

Trebst, A.V., H.Y. Tsujimoto and D.I. Arnon. 1958. Separation of light and dark phases in the photosynthesis of isolated chloroplasts. Nature 182: 351-355

Tremolieres, A. and M. Lepage. 1971. Changes in lipid composition during greening of etiolated pea seedlings. Plant Physiol. 47: 329-334

Tremolieres, A. and P. Mazliak. 1974. Biosynthetic pathway of α-linolenic acid in developing pea leaves. Plant Science Lett. 2: 193-201

Trosper, T. and C.F. Allen. 1973. Carotenoid composition of spinach chloroplast grana and stroma lamellae. Plant Physiol. 584-585

Tuquet, C., T. Guillot-Soplomon, M. De Lubac and M. Signol. 1977. Granum formation and the presence of phosophatidyl-glycerol containing trans-3-hexadecanoic acid. Plant Sci. Lett. 8: 59-64

Vagelos, P.R. 1974. Biosynthesis of saturated fatty acids. In: MTP International Review of Science, Biochemistry of Lipids (T.W. Goodwin, editor) Vol. 4: 99-140. Butterworths, London

Vance, W.A. and P.K. Stumpf. 1978. Fat metabolism in higher plants. The elongation of saturated and unsaturated acyl-CoAs by a stromal system from isolated spinach chloroplasts. Arch. Biochem. Biophys. 190: 210-220

Vernon, L.P., B. Ke, H.L. Mollenhauer and E.R. Shaw. 1969. Composition and structure of spinach subchloroplast fragments obtained by the action of Triton X-100. Prog. in Photosynthesis Res. I. (H. Metzner, editor) pp. 137-148

Vick, B. and H. Beevers. 1978. Fatty acid synthesis in endosperm of young castor bean seedlings. Plant Physiol. 62: 173-178

Vijay, I.K. and P.K. Stumpf. 1971. Fat metabolism in higher plants. XLVI. Nature of the substrate and the product of oleyl coenzyme A desaturase from Carthemus tinctorius. J. Biol. Chem. 246: 2910-2917

Visser, C.M. and R.M. Kellogg. 1978. Biotin. Its place in evolution. J. Mol. Evol. 11: 171-187

Volpe, J.J. and P.R. Vagelos. 1976. Mechanisms and regulation of biosynthesis of saturated fatty acids. Physiol. Rev. 56: 339-417

Wales, B. 1971. Plastid inheritance and mutations. See Benson, 1971, pp. 51-88

Walker, G.W.R., J. Dietrich, R. Miller and K. Kasha. 1963. Recent barley mutants and their linkages II. Genetic data for further mutants. Can. J. Genet. Cytol. 5: 200-219

Waring, A.J., R.W. Breidenbach and J.M. Lyons. 1976. In vivo modification of plant membrane phospholipid composition. Biochim. Biophys. Acta 443: 157-168

Weaire, P.J. and R.G.O. Kekwick. 1975. The synthesis of fatty acids in avocado mesocarp and cauliflower bud tissue. Biochem. J. 146: 425-437

Weinstein, J.D. and P.A. Castelfranco. 1978. Mg-protoporphyrin-IX and 5-aminolevulinic acid synthesis from glutamate in isolated greening chloroplasts. Arch. Biochem. Biophys. 186: 376-382

Wellburn, A.R. and F.A.M. Wellburn. 1972. Ultrastructural morphorgenesis in isolated etioplasts at high and low temperature and light flux. Proc. II Int. Congr. Photosynthesis (G. Forti, M. Auron and A. Melandri, editors) pp. 2329-2365. Dr. W. Junk N.V. Publishers, The Hague

Wessels, J.S.C. 1977. Fragmentation. See Amesz, 1971. Vol. 5: 563-573

Wessels, J.S.C. and M.T. Borchert. 1975. Studies on subchloroplast particles. Similarity of grana and stroma photosystem I and the protein composition of photosystem I and photosystem II particles. Proc. 3rd Int. Congress on Photosynthesis. (M. Avron, editor) Vol. 1: 473-484. Elsevier Scientific Publishing Company, Amsterdam, Oxford, New York

von Wettstein, D. 1958. The formation of plastid structures. Photochemical apparatus, its structure and function. Brookhaven Symp. Biol. 11: 138-159

von Wettstein, D., A. Kahn , O.F. Nielsen and S. Gough. 1974. Genetic regulation of chlorophyll synthesis analyzed with mutants in barley. Science 184: 800-802 von Wettstein, D., K.W. Henningsen, J.E. Boynton, G.C. Kannangara and O.F. Nielson. 1971. The genetic control of chloroplast development in barley. See Kirk, 1971a. pp. 205-223

Wharfe, J. and J.L. Harwood. 1978. Fatty acid biosynthesis in the leaves of barley, wheat and pea. Biochem. J. 174: 163-169

Williams, J.P. and P.A. Merrilees. 1970. The removal of water and nonlipid contaminants from lipid extracts. Lipids 5: 367-370

Williams, J.P., E.E. Simpson and D.J. Chapman. 1979. Galactolipid synthesis in *Vicia faba* leaves. III. Site(s) of galactosyl transferase activity. Plant Physiol. 63: 669-673

Williams, J.P., G.R. Watson, M.U. Khan and S. Leung. 1975. Galactolipid synthesis in *Vicia faba* leaves. I. Galactose, glycerol, and fatty acid labeling after $^{14}CO_2$ feeding. Plant Physiol. 55: 1038-1042

Williams, J.P., G.R. Watson and S.P.K. Leung. 1976. Galactolipid synthesis in *Vicia faba* leaves. Plant Physiol. 57: 179-184

Wintermanns, J.F.G.M. 1971. On the galactolipid composition of subchloroplast fragments. Biochim. Biophys. Acta 248: 530-535

Wong, E. 1976. Biosynthesis of flavonoids. See Bogorad, 1976. vol. 1: 464-526

Wood, H.G. and R.E. Barden. 1977. Biotin enzymes. Ann. Rev. Biochem. 46: 385-413

Woods, D.L. and K.W. Clark. 1971. Genetic control and seasonal variation of some alkaloids in Reed Canarygrass. Can. J. Plant Sci. 51: 323-329

Yamada, M. and Y. Nakamura. 1975. Fatty acid synthesis by spinach chloroplasts II. The path from PGA to fatty acids. Plant Cell Physiol. 16: 151-162

Yang, S.F., S. Freer and A.A. Benson. 1967. Transphosphatidylation by phospholipase D. J. Biol. Chem. 242: 477-484

Yao, V.J., C. Sterling and C.R. Stocking. 1972. Spinach chloroplast membranes after differential lipid extraction. Physiol. Plant 26: 355-363

Yoshida, Y. 1979. Nuclear dependency in chloroplast activities. Sci. Rep. Niigata Univ. Ser D (Biology) 16: 39-64

Zilkey, D. and D.T. Canvin. 1969. Subcellular localization of oleic acid biosynthesis enzymes in the developing castor bean endosperm. Biochem. Biophys. Res. Commun. 34: 646-653

Zill, L.P. and E.A. Harmon. 1962. Lipids of photosynthetic tissue. I. Silicic acid chromatography of the lipids from whole leaves and chloroplasts. Biochim. Biophys. Acta. 57: 573-582

Zucker, M. and H.T. Stinson, Jr. 1962. Chloroplasts as the major protein-bearing structures in Oenothera leaves. Arch. Biochem. Biophys. 96: 637-644